EFFECT OF ENVIRONMENTAL AND PHYSIOLOGICAL FACTORS ON N_2-FIXATION BY *INGA JINICUIL* AND *TRIFOLIUM* spp.

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P R O E F S C H R I F T

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE
WISKUNDE EN NATUURWETENSCHAPPEN
AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN, OP GEZAG VAN
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VOLGENS BESLUIT VAN HET COLLEGE VAN DECANEN
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Promotores: Prof. Dr. H.F. Linskens, University of Nijmegen.
Prof. Dr. R.H. Burris, University of Madison-Wisconsin.

Ne considérez pas les faits
selon vos arguments,
mais vos arguments d'après les faits.

To my parents and Betsy

CONTENTS

The number of species of the Family of the Leguminosae is estimated between 16.000 and 20.000, and these are found world wide (Allen and Allen, 1981). Approximately half of all the species are woody and those are mostly found in tropical regions (Vincent, 1974). Despite this large number of woody species, only recently the importance and the potentials of leguminous trees have been recognized. Besides the production of firewood (NAS, 1980), animal fodder (NAS, 1975, NAS, 1979) and the usefulness for soil improvement and reforestation (NAS, 1977), symbiotic nitrogen fixation with Rhizobium occurs. In Mexico, a legumious tree Inga jinicuil is found mainly in plantations where it provides shade for coffee trees (Avila and Goméz-Pompa, 1982). Coffee trees are planted at a density of approximately 1.200 per ha. whereas I. jinicuil has a density of around 200 per ha. (Roskoski et al. 1982). Roskoski (1981) showed that almost all the nodules of the I. jinicuil tree were found around the trunk of the coffee tree and not dispersed uniformly throughout the whole plantation. A possible explanation of this distribution pattern may be found in the way a coffee plantation is fertilized. More or less periodically, leaf litter around the coffee trunk is removed, fertilizers (N-P-K and sometimes micro-elements) are applied and leaf litter is replaced. Through the effect of these elements, a nodule concentration may occur at the place where the fertilizers were applied.

Effect of fertilizers on nodulation and N_2-fixation activity.

Effects of combined nitrogen on nodulation has been studied for a long period (Helz and Whiting, 1928, Mazé, 1898, Ritter, 1911). Numerous papers have been published in which the inhibitory effect of inorganic nitrogen on the formation of nodules is reported (Fred et al., 1932; Mahon and Child, 1979; Munns, 1968; Rabie and Kumazawa, 1979; Semu

and Hume, 1979; Tanner and Anderson, 1964, Tewari, 1965). However, differences in inhibition are observed depending upon the time and amount of the N application (Munns, 1968). Small amounts of N-fertilizer, so called 'starter', did lead to increase nodulation, N_2-fixation or yield (Bethlenfalvay et al., 1978; Dart et al., 1976; Gibson, 1976; Pankhurst, 1981; Pate and Dart, 1961). This effect probably arises because the plant has not yet formed effective N_2-fixing nodules, whereas N is required for the growth process. In this period of nitrogen hunger an application of combined nitrogen has a beneficial effect for yield. Besides nodulation, N_2-fixation is inhibited by N-fertilization (Manhart and Wong, 1980; Rigaud, 1976; Wilson, 1940). This is explained by the inhibition of nitrogenase synthesis when NH_4^+ is added (Brill, 1979).

The effect of K fertilization on nodulation and N_2-fixation has been studied less. Tewari (1965) reported that K had no effect on the formation of nodules by cowpea. In contrast, deMooy and Pesek (1966) reported that maximum nodulation and N_2-fixation by soybeans required very high levels of K. The same positive effect of K was observed by other investigators with soybean (Jones et al., 1977; Wu et al., 1969) and Vicia faba (Lynd et al., 1981). Mengel et al. (1974) found that plants well supplied with K showed higher concentrations of ^{15}N in different plant parts after ^{15}N-incorporation studies. Mengel et al. (1974) postulated a better carbohydrate supply from the leaves to the roots and nodules when K was adequate. This finding, according to the authors, was not sufficient to explain the stimulating effect of K on N_2-fixation. Feigenbaum and Mengel (1979) reported that a suboptimal K supply resulted in an inadequate provision of ATP and NADH, and this resulted in reduced plant growth and protein synthesis and reduced ^{15}N-incorporation.

The specific effect of P on nodulation and N_2-fixation also is not yet clearly understood. Helz and Whiting (1928)

reported a positive effect of P on nodulation by cowpea. This was confirmed for nodulation (Bouton et al., 1981; deMooy et Pesek, 1966; Jones et al., 1977; Zaroug and Munns, 1979) and N_2-fixation (Bouton et al., 1981; Döbereiner, 1977; Hutchings, 1936). Cassman et al. (1981) and Whitney (1977) reported that species such as cowpea and stylo (Stylosanthes guyanensis) were more tolerant to P stress than soybean. The same phenomenon was observed for Rhizobium strains (Keyser and Munns, 1979, Munns, 1979).

Molybdenum is required for N_2-fixation and is an essential element for one of the two proteins which form the nitrogenase enzyme complex (Yates, 1980). In most areas no Mo fertilization is needed, because the requirements for Mo for N_2-fixation are low and enough Mo is present in the soil. However, in some tropical soils, Mo deficiency appears and Mo fertilization has shown a beneficial effect (Lie et al., 1979). It appears that for N_2-fixation Mo requirements are greater than for nodulation (Mulder et al., 1959) and more Mo is required for plants depending on N_2-fixation than for plants depending on soil nitrogen (Parker and Harris, 1977).

Magnesium is an essential element for the growth of free living Rhizobium (Norris, 1959). Further, during the reduction of N_2 two molecules of MgATP bind with Fe-protein (Burris et al., 1980). Despite these requirements for Mg, not much is known about the effects of Mg fertilization for nodulation and N_2-fixation under field conditions.

Diurnal and seasonal variations in N_2-fixation rates.

After the introduction of the acetylene reduction assay (Dilworth, 1966, Schöllhorn and Burris, 1967), this method has become very popular for calculation of N_2-fixation rates by all N_2-fixing organismn (Turner and Gibson, 1980). One of the reasons for this popularity is the short incubation time required. Due to this short incubation period, possible diurnal variation in N_2-fixation will not be noticed with a single assay. When the importance of

biological N_2-fixation input into an eco-agricultural system has to be calculated, daily and seasonal variations in N_2-fixation rates should be known. A diurnal cycle in N_2-fixation has been reported for plants such as Leucaena leucocephala (Högberg and Kvarnström, 1982) and Trifolium repens (Masterson and Murphy, 1976). Differences between day and night N_2-fixation rates are in the order of 1.5-3 : 1 (Pate, 1976) and probably are caused by a lower supply of photosynthates during the night than during the day (Hardy et Havelka, 1976). However, differences in soil and air temperature and humidity between day and night also may affect N_2-fixation (See Minchin et al., 1981).

Acetylene reduction versus $^{15}N_2$-fixation.

Acetylene is not the physiological substrate for the nitrogenase enzyme, and a conversion of acetylene reduced to N_2 fixed has to be made. Where for the reduction of N_2 $6e^-$ and 12 ATP are required, 2 e^- and 4 ATP are required for the reduction of acetylene to ethylene (Mulder, 1975). This stoichiometric comparison leads to the theoretical ratio of 3 for C_2H_4/N_2. Burris (1974), however, pointed out that this ratio is often not correct and that the appropriate ratio should be established. With the use of the ^{15}N isotope it is possible to measure the real N_2-fixing rate. When under the same incubation conditions $^{15}N_2$ and, seperately, C_2H_2 are employed as substrates, the exact ratio can be established (Burris, 1974).

H_2 evolution and Relative Efficiency.

The reason that the C_2H_4/N_2 ratio of 3 is not observed is because of the production of H_2 during the reduction of N_2. The reduction of protons during N_2 reduction appears to be obligatory for the system (Schrauzer, 1976), and for each mol of N_2 reduced at least one mol of H_2 will be produced (Rivera-Ortiz and Burris, 1975). The production of H_2 requires ATP (Ljones et Burris, 1972;

Hadfield and Bulen, 1969). A pC_2H_2 of 10 kPa only partly blocks the production of H_2 (Drevon et al., 1982; Gibson and Alston, 1981; Peters et al., 1977 Rivera-Ortiz and Burris, 1972) and during the acetylene reduction assay the total electron flux through nitrogenase is not measured through the production of ethylene.

Furthermore, C_2H_2 does not block H_2 uptake by hydrogenase (Emerich et al., 1979; Houchins and Burris, 1981; Peterson and Burris, 1978), and the H_2 produced can be recycled. All the N_2-fixing organisms possess a hydrogenase (Robson and Postgate, 1980), but not all hydrogenases are active or show only a low activity . Hydrogenases in legume root nodule bacteroids has been studied by Dixon (Dixon, 1967; Dixon, 1968; Dixon, 1972), and he postulated three functions for hydrogenase: 1. Through the oxidation of H_2 some of the energy lost during the reduction of protons is recovered. 2. By removal of H_2 the inhibitory effect of H_2 during N_2-fixation is decreased. 3. During the oxidation of H_2 a consumption of O_2 takes place and hydrogenase thus acts as a protector for nitrogenase which is O_2 labile.

When an active hydrogenase is present, no or only low amounts of H_2 will evolve. The rate of H_2 evolution has been used for calculation of the relative efficiency of the nitrogenase system. This relative efficiency has been defined by Schubert and Evans (1976) as follows:

$$\text{Relative Efficiency (R.E.)} = 1 - \frac{\text{Rate of } H_2 \text{ production in air}}{\text{Rate of } C_2H_4 \text{ production}}$$

Evans and coworkers reported that 30% (Evans et al., 1978) and even 40 to 60% (Schubert and Evans, 1976) of the electron flux through nitrogenase is used for the production of H_2. Similar results were found elsewhere (Bethlenfalvay and Phillips, 1979; Bethlenfalvay et al., 1978[b]). From a more practical point of view is the question whether plants possessing a more efficient nitrogenase produce higher yields. In a number of papers Evans and coworkers showed that

soybeans possessing efficient N_2-fixing rhizobia did produce higher yields than those soybeans inoculated with Hup^- rhizobia (Albrecht et al., 1979; Hanus et al., 1981; Schubert et al., 1978; Zablotowicz et al., 1980). They found a 15.7% increase in plant dry weight and a 26.6% increase in total nitrogen. Pahwa and Dogra (1981) confirmed the positive effect of Hup^+ possessing Rhizobium on yield of mungbean, Miller and Sirois (1982) found a poor correlation between nitrogenase efficiency and plant yield by Medicago sativa but a high correlation between nitrogenases activity x efficiency and yield. Nelson and Child (1981) did not find significant differences between Pisum sativum plants inoculated with either Hup^+ or Hup^- strains on yield or total nitrogen. Gibson et al. (1981) however, observed an inverse relationship between R.E. and symbiotic effectiveness (yield) by Trifolium subterraneum. Rainbird et al. (1983) came to the conclusion that cowpeas nodulated with a high or a low H_2 evolving Rhizobium strain did not differ in dry matter production, seed yield, and nitrogen fixed. However, they found that H_2 evolving symbiosis lost more CO_2 during respiration than the Hup^+ possessing symbiosis. Further they found that the H_2 evolved/N_2 fixed ratio varied markedly during ontogeny, and thus changed the relative efficiency, a phenomenon earlier reported by Bethlenfalvay and Phillips (1977). A general consensus about the beneficial effect of the use of Hup^+ Rhizobium strains on yield and total N has not been reached as became clear again during a recent conference (Schubert, 1982).

The use of ^{15}N-enriched and ^{15}N-depleted N-compounds.

Currently most R.E. are based on acetylene reduction rates. As pointed out earlier, C_2H_2 is not the physiological substrate for nitrogenase and this can lead to problems. Knowles (1981) mentioned several problems associated with acetylene as a substrate for measuring nitrogen fixation. 1. Because C_2H_2 largely suppresses

the nitrogenase-catalyzed H_2 production it is not known what percentage of the electron flux goes to the reduction of N_2 and what percentage to the reduction of protons. This leads to problems when the rate of C_2H_4 production is converted to the rate of N_2-fixation (Hudd et al., 1980). 2. The possible positive effect of recycling H_2 through hydrogenase uptake and providing ATP to nitrogenase is largely eliminated when C_2H_2 is used as a substrate and this may lead to a decrease of activity. 3. Oxidation of C_2H_4 is inhibited by C_2H_2 and the C_2H_4 detected may not only be a product of the nitrogenase (Witty, 1979) but also may be endogenously formed C_2H_4.

Another technical problem with the acetylene reduction assay is the possible leakage or diffusion of gases out of the incubation vessel. Currently, glass vessels (Bergersen, 1970; Hardy et al., 1968) or air tight chambers (Montange et al., 1981; Lethbridge et al., 1982; Wych and Rains, 1978) have been used. In field studies, when large samples will be analyzed, plastic bags can be used (Turner and Gibson, 1980). However, leakage through the plastic film will occur (Burris, 1974, Rogers et al. 1956) and the absolute rate of diffusion of the gases employed should be established especially for long term incubation.

These problems with the acetylene reduction method make it desirable to use other methods as well for the calculation of the percentage of N fixed. Some such methods were well known before the introduction of acetylene reduction assay. The oldest method known is comparing total nitrogen of the plants. Using this method Hellriegel and Willfarth (1888) were the first to establish clearly the presence of biological N_2-fixation. This method is time consuming and not very accurate. When the isotope ^{15}N became available, Burris and Miller (1941) showed that there was no detectable discrimination between ^{15}N and ^{14}N incorporation during N_2-fixation in Azotobacter vinelandii. Later the use of ^{15}N-enriched N-compounds were used in field studies (Boddey and

Chalk, 1983; Domenach et al., 1979; Feigenbaum and Hadas, 1980; Rennie et al., 1982; Ruschel et al., 1979; Wagner and Zapata, 1982). Enriched ^{15}N-compounds were applied in the field and after the growth period plants were analyzed for atom % ^{15}N. Differences in atom % ^{15}N between the N_2-fixing plant and a control non-N_2-fixing plant could give information about the % of total N derived from N_2-fixation. A great advantage of the method is that the incubation time is prolonged to the whole growth cycle of the plant and all the environmental and physiological factors affecting N_2-fixation are included in the atom % ^{15}N later found in the plants analyzed. A problem, however, is the choice of the non-N_2-fixing control plant. Several investigators used a non-N_2-fixing as control plant (Ham, 1977; Deibert et al., 1979; Talbott et al., 1982), others a cereal (Ruschel et al., 1982; Fried and Broeshart, 1981; Wagner and Zapata, 1982), uninoculated soybeans (Rennie et al., 1982) or grasses (Broadbent et al., 1982; Domenach et al., 1979; Williams et al., 1977). Assumptions are made that no differences occur between test plants and control plants in root activity in taking up N, that root growth is similar and that no transfer on N between test plants and control plants takes place (Deibert et al., 1979; Rennie, 1979). Associative N_2-fixation should not occur or should be equal for all the plants tested (Boddey and Chalk, 1983), and there should be no spatial variability in atom % ^{15}N of the soil nitrogen (Broadbent et al., 1980). The best control plant will be a non-N_2-fixing isoline but for the most species no such control plants are available.

Instead of ^{15}N-enriched compounds, ^{15}N-depleted (i.e., ^{14}N-enriched) N compounds can be used. Atmospheric N_2 contains an atom % ^{15}N of approximately 0.365 % (Nier cf Delwiche and Steyn, 1970). Plants using soil- or atmospheric N should show about the same atom % ^{15}N when ^{15}N-depleted N-compounds are used as N-source, plants utilizing those compounds shown an atom % ^{15}N less than the

atom % ^{15}N of atmospheric N_2. The observed differences in atom % ^{15}N between N_2-fixing and non-N_2-fixing plants will be smaller than when ^{15}N-enriched N compounds are used. But with the availability of sensitive isotope ratio mass spectrometers, differences in atom % ^{15}N should be large enough to detect significant differences between test and control plants. The main difference between ^{15}N-enriched and ^{15}N-depleted N compounds is the price. Whereas ^{15}N-enriched compounds are expensive, the ^{15}N-depleted compounds are relatively inexpensive. But the use of ^{15}N-depleted compounds in nitrogen fixation studies has been very limited. To my knowledge only Broadbent and Carlton (1980) have described this method and used it in field trials (Broadbent et al., 1982).

^{15}N-natural abundance of N_2-fixing plants.

With the availability of more precise isotope ratio mass spectrometers another method for calculation of the percentage of total N derived from biological N_2-fixation has been used. In 1955 Hoering (Hoering, 1955) reported the occurrence of variations in ^{15}N natural abundance in plant tissue and soil. When compared with the atom % ^{15}N of atmospheric N_2, white clover showed a slightly negative value of -6.5 (based on $^{15}N/^{14}N$ ratio), whereas elm leaves showed a positive value of +1.9. Further studies led Delwiche and Steyn (1970) to the suggestion that the N-isotope composition of N_2-fixing plants and other non-N_2-fixing plants could give information about the source of nitrogen. During the process of N_2-fixation a small discrimination against ^{15}N occurs (Rennie et al., 1976; Mariotti et al., 1980; Delwiche and Steyn, 1970). Due to this small discrimination, a plant dependent on soil N should show a higher $^{15}N/^{14}N$ ratio than an N_2-fixing plant, especially when it is known that soil N is slightly but variably enriched in ^{15}N as compared with atmospheric N_2, (Bremner and Tabatabai, 1973; Cheng et al., 1964; Feigin et

al., 1974[a]; Feigin et al., 1974[b]; Shearer et al., 1978). Amarger et al. (1977) used this method to measure the percentage of total N derived from N_2-fixation. For that experiment Lupinus luteus var. sulfa was the test plant and a non-inoculated Lupinus luteus var. sulfa was the control plant. Both plants were grown in a greenhouse under a controlled environment and different levels of nitrate were applied. This choice of a control plant is possible in greenhouse studies but is unsuitable in field studies. In a field study testing soybeans, the assumption was made that plants growing in non-inoculated plots did not from nodules and as such could be used as control plants (Amarger et al., 1979). Problems with the choice of a control plant are similar when ^{15}N-enriched N compounds are used. However, some additional problems arise with this method for calculating the percentage of N derived form N_2-fixation. Delwiche et al. (1979) reported that different plant families showed different $^{15}N/^{14}N$ ratios. Further, the ^{15}N-distribution of an N_2-fixing plant is not uniformly distributed through the whole plant and is dependent on the age of the plant (Amarger et al., 1979; Mariotti et al., 1980; Rennie et al., 1976; Shearer et al., 1980). The ^{15}N-natural abundance of nodules appears to increase with time when compared with the ^{15}N-natural abundance of the whole plant (Shearer et al., 1980), whereas the ^{15}N-natural abundance of the whole plant appears to decrease with time (Kohl and Shearer, 1980). An explanation for the increase in ^{15}N-natural abundance of nodules may be found in the form of the compound in which N is transported to the rest of the plant. According to Shearer et al. (1980) ureide transporting nodules show a higher ^{15}N-natural abundance than amide transporting nodules. Another cause of different values for ^{15}N-natural abundance could be N_2-fixing efficiency (Shearer et al., 1982). Ureide transporting nodules with a higher N_2-fixation efficiency should show a higher ^{15}N-natural abundance than nodules with a lower N_2-fixation

efficiency. No effect or correlation was found for amide transporting nodules.

Several investigators reported a normal isotope effect during N_2-fixation and/or N transport through the plant (Bardin et al., 1977; Delwiche and Steyn, 1970; Mariotti et al., 1980). This normal isotope effect indicates that the heavier ^{15}N atom is discriminated against in favor of the lighter ^{14}N atom. This would lead to a lower $^{15}N/^{14}N$ ratio in a N_2-fixing plant than in atmospheric N_2. Kohl and Shearer (1980), however, found an inverse isotope effect with Glycine max and Trifolium pratense grown hydroponically in an N-free medium. When N_2-fixation measurements are carried out using the natural ^{15}N-abundance method, this inverse isotope effect can significantly influence the outcome of the calculations, especially when observed differences in $^{15}N/^{14}N$ ratios between test plants and atmospheric N_2 or control plants are small.

Scope of the investigation.

The influence of fertilization on N_2-fixation by Inga jinicuil, a shade tree in Mexican coffee plantations, has been studied. For calculation the annual input of biological fixed N_2 into the agro-ecosystem, diurnal and seasonal fluctuations in N_2-fixation were calculated. Because N_2-fixation activity was measured indirectly using the acetylene reduction method, ^{15}N-incorporation studies also were carried out and the C_2H_4/N_2 ratios were established.

Possible diffusions of gases through plastic bags, used for acetylene reduction assays or $^{15}N_2$-fixation studies, were measured. Efforts were made to establish a more exact C_2H_4/N_2 ratio without the direct use of $^{15}N_2$ but including H_2 evolution rates. Other methods were employed to calculate the percentage of total N derived from biological N_2-fixation. The possible use of depleted ^{15}N-compounds was investigated. The method based on ^{15}N natural

abundance was used and possible differences in atom % ^{15}N, caused by different Rhizobium strains, was studied.

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CHAPTER 1.

Nodulation and N_2 fixation by *Inga jinicuil*, a woody legume in coffee plantations.

II. Effect of soil nutrients on nodulation and N_2 fixation.

NODULATION AND N_2 FIXATION BY *INGA JINICUIL* A WOODY LEGUME IN COFFEE PLANTATIONS

II EFFECT OF SOIL NUTRIENTS ON NODULATION AND N_2 FIXATION

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KEY WORDS

Acetylene reduction Coffee plantation *Inga jinicuil* Nodulation Nutrients Woody legume

SUMMARY

The effect of soil nutrients on nodulation by *Inga jinicuil* a leguminous tree used for shade in Mexico in coffee plantations is discussed Nodulation and C_2H_2 reduction of *I jinicuil* seedlings grown in soil taken at different distances from coffee trunks is described Nodule biomass and activity are compared to the nutrient content of soils within various distances of coffee trunks Seven coffee plantations that employ *I jinicuil* for shade were examined with respect to nodule biomass C_2H_2 reducing activity and soil characteristics

Highest nodule biomass was observed in soils with high levels of available phosphorus High nitrogen content of the soil on the other hand appeared to be correlated with low nodule biomass Potassium and magnesium while apparently having little effect on nodule biomass seem to be positively correlated with C_2H_2 reduction

INTRODUCTION

Root nodules of *Inga jinicuil* Schlechter a leguminous tree used for shade in Mexican coffee plantations occurred around the base of coffee trees Beyond a distance of 40 cm from coffee trunks the biomass of nodules decreased sharply

One explanation for the distribution pattern of root nodules may be related to the way the coffee plantation is fertilized Three times a year the leaf litter around each coffee tree is removed and N P K fertilizer is applied in a ring within a radius of 40 cm from the trunk Then the leaf litter is replaced along with additional litter from the surrounding area

Since the effect of nitrogen, phosphorus and potassium on nodulation and N fixation of several crop legumes is well established[1,2,4,5,6,10,11,15,16,17,18] we

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hypothesized that *I jinicuil* nodules might be more abundant under coffee trees as a result of nutrient additions via fertilizers The present study describes the correlation between nodulation and C_2H_2 reducing activity of *I jinicuil* and the chemical composition of the soil

MATERIALS AND METHODS

The study was conducted in 1978 1979 in and around Xalapa state of Veracruz Mexico Xalapa is located at 19 27 latitude north 96 57 longitude west and 1295 meters above sea level Winters are wet and cool summers semi hot and humid All coffee sites studied contained an average of 2500 coffee trees and 225 *I jinicuil* trees ha $^{-1}$

Pot experiments

Three coffee trees were randomly chosen in coffee plantation Orduna 1 Soil to a depth of 20 cm was gathered at distances of 0–30 cm 30 60 cm and 60 100 cm respectively from each coffee trunk These soils were composited by distance and then mixed with sand at 1 1 ratio of 1 1 to increase drainage

Seeds of *I jinicuil* were collected from the same site in August 1978 These were treated with Mercaptan to prevent fungal infection and stored in wet sand at 8 C for one month The seeds were then planted in plastic bags 10 cm in diameter and 15 cm height each containing approximately 2 kg of one of the sand soil mixtures Bags were placed in partial shade in the Botanical Garden of Xalapa In addition some seeds were planted directly in soil of the Botanical Garden adjacent to the bags Plants were watered when necessary

1 our and a half months after planting 3 plants from each of the four test soils were randomly selected The root system of each plant including the nodules was placed in a separate glass jar and assayed for C_2H_2 reducing activity [3] Following assay nodules leaves and stems were weighed after drying for 2 days at 80 C For the next 4 months three plants from each test soil were harvested monthly and analyzed for C H reduction At 9 and 10 months after planting 5 instead of 3 plants were assayed

At the time of the 4 5 and 8 5 month assays chemical and physical analyses of soils in which the analyzed plants had grown were conducted The pH of a 1 1 soil water paste was determined Additional soil was air dried passed through a 0 4 mm sieve further dried for 2 days at 105 C and then analyzed for total Kjeldahl nitrogen available phosphorus using the method of Bray and organic matter content by the Walkley Black method[9] Calcium magnesium and potassium were determined by atomic absorption spectrophotometry of ammonium acetate extracts[9]

Field experiments

Forty soil samples 20 cm in diameter and 20 cm in depth were randomly taken at various distances from coffee trunks in site Orduna 1 An additional 11 samples were also taken to the depth at which no more nodules could be found usually 5 to 10 cm Sampling distances were 10 30 50 90 and 110 cm from the coffee trunks Three nodules removed from each soil sample at the time of collection were assayed separately for C H_2 reduction as described above A sub sample of the soil was analyzed for pH K P Mg Ca total nitrogen and organic matter The nodules present in the remaining soil were collected and weighed after drying for 2 days at 80 C

In addition soil samples and nodules were collected in seven other coffee plantations around Xalapa that employ *I jinicuil* trees for shade About 24 soil samples 20 cm in diameter and 20 cm in depth were taken at 10 30 50 90 and 100 cm respectively from coffee trunks in each site and C H

reduction activity of nodules was measured as described previously Soil samples were taken to the laboratory where nodules were separated from the soil Three soil samples from each site were analyzed for total nitrogen K Mg Ca P pH and organic matter

RESULTS AND DISCUSSION

Pot experiments

Nodules first appeared on seedlings grown in bags (Fig 1) Plants grown in soils 30–60 cm and 60–100 cm had nodules 4 5 months after planting, plants in soil 0–30 cm 5 5 months Nodules were observed on seedlings planted directly in the

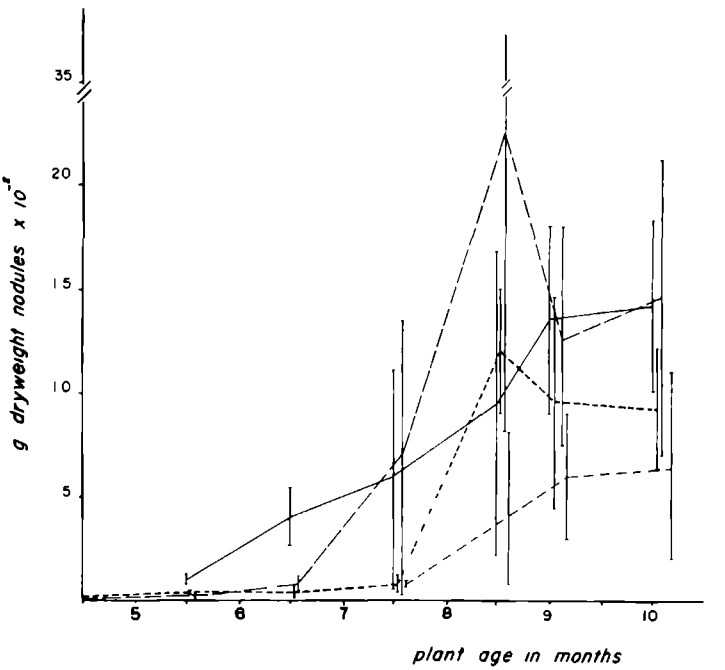

Fig 1 Nodule biomass of *Inga jinicuil* seedlings vs time since planting Values shown are means ± 1s Lines are — soil 0–30 cm soil 30–60 cm soil 60–100 cm Botanical garden soil

Table 1 Relationship between leaf biomass and nodule biomass

Months after planting	Regression equation*	r**
6 5	f(x) = 0 08x − 0 06	0 73
7 5	f(x) = 0 15x 0 10	0 70
8 5	f(x) = 0 32x − 0 23	0 79
9 5	f(x) = 0 22x 0 14	0 96
10 0	f(x) 0 16x − 0 06	0 78

* f(x) is nodule biomass where x is leaf biomass
** correlation coefficient

garden soil only after 7 5 months Nodule biomass reached a maximum 8 5 months after planting for seedlings in soils 30–60 cm and 60–100 cm and 9 months after planting in soil 0–30 cm

Nodule biomass was highly correlated with leaf biomass throughout the experiment (Table 1) The highest nodule biomass per gram leaf biomass occurred 8 5 months after planting Since the processes of nodulation and nitrogen fixation require large amounts of photosynthate[12], the occurrence of maximum nodule biomass at the time of maximum leaf biomass, when the leaves are young and have the highest photosynthetic potential seems reasonable

Phosphorus has been reported as essential for optimal nodulation of legumes[6] Therefore, the significantly higher biomass of nodules in soil 0–30 cm as compared to soils 30–60 cm and 60–100 cm ($p = 05$) during the first 6 5 months, may be due to the higher P level in soil 0–30 cm (Table 2) Similarly the lower level of P in the garden soil may explain the absence of nodules during the first 7 5 months A second reason for the late development of nodules in the garden soil may be the

Table 2 Soil analyses of the four test soils*

Distance from coffee trunk cms	pH	O M**	% N	ppm K	ppm Ca	ppm Mg	ppm P
0 30	4 8 + 0 1	3 1 ± 0 4	0 16 ± 0 02	192 ± 32	457 ± 12	98 ± 25	64 ± 2
30 60	5 2 ± 0 0	2 6 ± 0 3	0 13 ± 0 02	277 ± 15	557 + 17	124 ± 13	54 ± 3
60 90	5 8 + 0 1	2 3 ± 0 1	0 12 ± 0 01	220 ± 11	788 ± 98	39 ± 14	44 ± 3
Botanical garden	5 5 ± 0 0	5 2 ± 0 3	0 33 ± 0 01	382 ± 39	1145 + 72	220 ± 41	29 ± 4

* Values shown are the mean ± ls, of three bags harvested per soil 6 5 months after planting
** O M organic matter

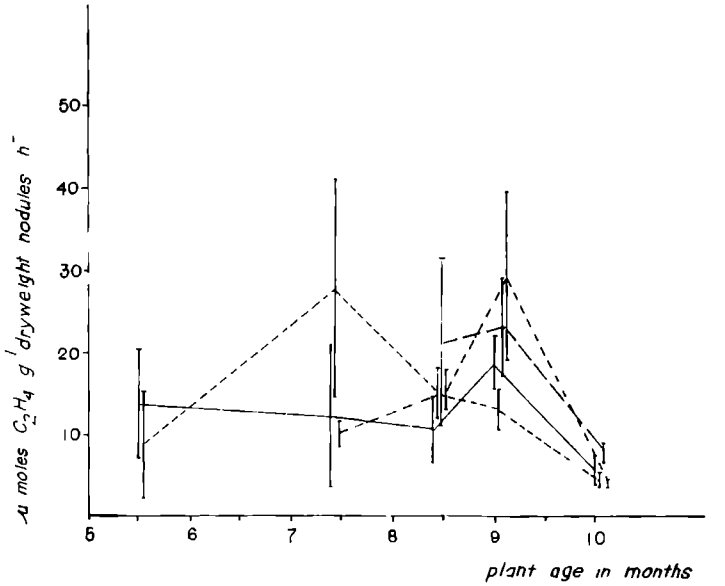

Fig 2 C_2H_2 reduction values of nodules from *Inga jinicuil* seedlings vs time since planting Values shown are means ± 1s Lines are Lines are — soil 0-30 cm - soil 30-60 cm – soil 60–100 cm from coffee trunks Botanical garden soil

high nitrogen content of this soil (Table 2) which may have inhibited nodulation[3,7,14]

During the last sampling period the nodule biomass did not change, while nitrogen fixing activity decreased for all four soils (Fig 2) Since the nodules in the garden soil first appeared three months later than those in the other three soils nodule senescence, at least for nodules in the garden soil, seems unlikely A seasonal effect such as leaf senescence may be one explanation for this phenomenon

Field experiments

Data on nodule biomass and various soil characteristics for the forty soil samples from site Orduña 1 were analyzed using a multiple step-wise regression program

BMDP2R[8] No significant relationship was found between nodule biomass and the chemical characteristics of the soils in which the nodules occurred. Furthermore, no significant relation existed between C_2H_2 reduction activity of the nodules and the chemical characteristics of the soil.

The same regression analysis, performed on data for the 11 samples, taken only to the depth at which nodules occurred, revealed that nodule biomass and the phosphorus content of soils were highly correlated ($p = .05$). This finding agrees with other reports[5,6], on the importance of phosphorus for nodulation and/or nodule growth.

C_2H_2 reduction activity for the thirty-one nodule samples, extracted from the 11 soil samples, was found to be highly correlated with Mg and K levels of the soil.

These results suggest that shallow sampling, 5 to 10 cm, was better for comparing the influence of nutrient levels on nodulation and C_2H_2 reduction of *I. jinicuil*, since it included only the area immediately adjacent to the nodules. Sampling to a greater depth, as was done for the forty samples, undoubtedly encompassed soil horizons with nutrient levels considerably different from that in which the nodules occurred.

Nodules were found in all seven coffee sites examined. However, the abundance of nodules varied widely from site to site. Orduña I had the highest mean nodule biomass per sample, and the largest number of samples with nodules. The lowest site, with respect to the above two parameters, was Jilotepec (Table 3).

Analysis of variance-BMDP2V[8] revealed that a significant difference in nodule biomass between the seven sites ($F = 5.89$, df 6,173). Using Duncans multiple-range test to compare mean nodule biomass[19] showed that Orduña I had a significantly higher mean nodule biomass than the other six sites ($p = .05$). However, no significant differences were found among the six sites.

Table 3. Occurrence of *Inga jinicuil* nodules in seven coffee plantations

Plantation	x g dry weight nodules sample^{-1}	% samples with nodules
Teocelo	0.0386	45.0
Xalapa	0.1638	12.5
Orduna 2	0.2207	45.8
Santa Barbara	0.0163	25.0
Chichitla	0.1065	54.2
Jilotepec	0.0025	4.2
Orduña 1	3.1348	92.5

Table 4 Nutrient levels and pH of soils from seven coffee plantations*

Plantation	pH	% N	ppm K	ppm Ca	ppm Mg	ppm P
Teocelo	5.6 ± 0.1	0.33 ± 0.10	144 ± 69	1255 ± 473	144 ± 69	66 ± 25
Xalapa	5.0 ± 0.1	0.80 ± 0.10	252 ± 42	1525 ± 219	124 ± 24	18 ± 3
Orduña 2	4.9 ± 0.1	0.47 ± 0.06	250 ± 38	637 ± 117	56 ± 14	18 ± 4
S. Barbara	5.2 ± 0.1	0.31 ± 0.05	190 ± 26	725 ± 185	85 ± 15	35 ± 1
Chichitla	4.8 ± 0.2	1.33 ± 0.23	500 ± 204	2505 ± 819	259 ± 56	13 ± 4
Jilotepec	5.9 ± 0.1	2.01 ± 0.10	177 ± 23	33945 ± 24946	540 ± 99	59 ± 28
Orduna 1	4.7 ± 0.1	0.55 ± 0.02	420 ± 23	451 ± 55	56 ± 4	105 ± 7

* Values shown are the means ± ls_x

The highest level of phosphorus was found in soil samples from Orduña 1 (Table 4) Analysis of variance further showed that significant differences existed between the seven sites with respect to the phosphorus ($F = 7.17$, df 6,151), and nitrogen ($F = 40,40$, df 6,50) levels of their soils Duncans test demonstrated that Orduña 1 had significantly higher phosphorus levels than Santa Barbara, Orduña 2, Xalapa, and Chichitla, and significantly lower nitrogen levels than Jilotepec, Chichitla, and Xalapa These data further establish the positive effect of phosphorus and the negative effect of nitrogen on nodulation and/or nodule growth of *I. jinicuil*

Table 5 C_2H_2 reduction activity of *I. jinicuil* nodules collected from different coffee plantations

Plantation	µmoles C_2H_2 reduced g^{-1} nodules h^{-1}*
Teocelo	0.75 ± 0.22
Orduña 2	0.68 ± 0.06
Santa Barbara	0.69 ± 0.13
Chichitla	0.43 ± 0.09
Orduña 1	0.98 ± 0.26

* Values shown are means ± ls_x

C_2H_2 reduction activity was highest for Orduña 1 and lowest for Chichitla (Table 5), but these differences were not statistically significant. In Xalapa and Jilotepec no active nodules were found.

GENERAL DISCUSSION

In both the pot and field studies, high nodule biomass was associated with high levels of available phosphorus. This suggests that phosphorus acts to stimulate nodulation and or nodule growth of *P. jimicuil*. On the other hand, high nitrogen levels were usually associated with low nodule biomass. Since both nitrogen and phosphorus are applied during fertilization in the sites studied, it appears that at the levels of fertilizer used in some coffee plantations, the stimulatory effect of phosphorus overrides the inhibitory effect of nitrogen.

C_2H_2 reduction activity was correlated with magnesium and potassium. The scarcity of information on the effect of these two elements on nitrogen fixation makes it somewhat difficult to evaluate our results. Additional experimental studies are aimed at addressing this question.

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CHAPTER 2.

Nodulation and N_2 fixation by Inga jinicuil, a woody legume in coffee plantations.
III. Effect of fertilizers and soil shading on nodulation and nitrogen fixation (acetylene) of I. jinicuil seedlings.

Plant and Soil 72 95–105 (1983) Ms 5121

Nodulation and N_2 fixation by *Inga jinicuil*, a woody legume in coffee plantations

III Effect of fertilizers and soil shading on nodulation and nitrogen fixation (acetylene reduction) of *I jinicuil* seedlings

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Key words Acetylene reduction *Inga jinicuil* Nitrogen fixation Nodulation Nodule biomass Nutrients Shading Tropics Woody legume

Summary A pot experiment was conducted to assess the effects of different fertilizers and soil shading on nodulation and acetylene reduction of *Inga jinicuil* seedlings Initially seedlings produced maximum nodule biomass when grown with high levels of phosphorus but reduced the most acetylene under intermediate phosphorus fertilization These response differences however gradually diminished with age being negligible when the seedlings were a year old Nitrogen fertilization inhibited nodulation and acetylene reduction throughout the experiment Potassium did not significantly affect nodulation but low levels of potassium stimulated and high levels inhibited acetylene reduction activity relative to unfertilized control plants Neither magnesium nor molybdenum affected nodulation or acetylene reduction Soil shading resulted in decreased nodule biomass and less nitrogen fixing activity during summer months However the data suggest that shading may favour nitrogen fixation in colder periods by moderating soil temperatures

These results confirm findings from an earlier field study and show that nodulation and nitrogen fixing activity by leguminous trees is influenced by the types and amounts of nutrients supplied This suggests that the quantity of nitrogen fixed by leguminous shade trees in coffee plantations may be amenable to manipulation through simple management techniques

Introduction

Recently interest in leguminous trees especially in tropical regions of the world has increased[15,16] This is due to the large number of Leguminosae found in the tropics[7] and the potential utility of these species to provide needed resources and raw materials Tree legumes are or can be used for animal fodder human food firewood erosion checks revegetation and shade for crops and or cattle[16,12] In addition many leguminous trees fix nitrogen[1] a macro-element for plant growth thereby increasing the nitrogen content of ecosystems in which they occur Despite this fact few studies have examined the factors that affect nitrogen fixation by tree legumes

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Previously we reported the occurrence of nodules in a Mexican coffee plantation where leguminous tree *Inga jinicuil* Schlechter is used for shade[19]. Furthermore we estimated that nitrogen fixation by this species constituted a significant nitrogen input to the coffee agro-ecosystem[19] and suggested that nodule biomass and activity were influenced by soil nutrients[1]. The data indicated that high soil phosphorus and low soil nitrogen were correlated with high nodule biomass and that nitrogen-fixing activity was correlated with potassium and magnesium.

In this paper we present the results of a study to characterize under more controlled conditions the effects of phosphorus, nitrogen, potassium, magnesium, molybdenum and soil shading on nodulation and nitrogen fixing activity (acetylene reduction) of *I. jinicuil* seedlings.

Materials and methods

The study was conducted at the Instituto Nacional de Investigaciones sobre Recursos Bioticos in Xalapa, Mexico (19° 28' latitude north, 96° 57' longitude west, and 1295 meters above sea level). Winters are wet and cool, summers are semi-hot and humid[1].

During the summer of 1979, *I. jinicuil* seeds were collected from a coffee plantation near the Instituto and planted in plastic cups filled with soil from the Botanical Garden. The soil is a volcanic ash-derived clay with a high percentage of allophanic clays. It was obtained by first removing the top 20 cm of soil, which was discarded. The underlying soil to a depth of 70 cm was then thoroughly mixed to yield a homogeneous soil.

Chemical and physical analyses of the soil was conducted. The pH of a 1:1 soil:water paste was determined. Additional soil was dried, passed through a 0.4 mm sieve, further dried for 2 days at 105° C, and then analyzed for total Kjeldahl nitrogen, available phosphorus using the method of Bray, and organic matter content by the Walkley-Black method[6]. Calcium, magnesium, and potassium were determined by atomic absorption spectrophotometry of ammonium acetate extracts. The characteristics of this soil were as follow: pH = 5.05, percent total N = 0.273, available ppm P = 6.0, ppm K = 175, ppm Ca = 769, ppm Mg = 168.

After 90 days the seedlings were transplanted from the cups to plastic bags containing 7 kg of soil. Holes were punched into the bottom of each bag to facilitate drainage. To prevent the emergence of roots through these holes, each bag was inserted into a second bag whose drainage holes were offset to those of the inside bag. Bags were placed outdoors in the full sunlight in one continuous line. Placement of an individual bag within the line was done randomly.

Fertilizers were applied one week after transplanting. A factorial design was employed with treatments allocated to bags in a completely random manner. Treatments were four levels of superphosphate (0.0, 3.1, 6.3, or 10.5 g/bag which were equal to 0, 105, 265, or 525 kg P/ha) with 0.0, 0.95, or 4.6 g urea/bag (0, 96, or 482 kg N/ha) or 0.0 or 3.7 g $MgCO_3$ – 2.3 g $MgCl_2 \cdot 6H_2O$/bag (0 or 0.94 – 6.9 kg Mg/ha) or 0.0, 0.4, or 2.0 g KCl/bag (0, 52, or 260 kg K/ha) or 0.0 or 0.5 g $Na_2MoO_4 \cdot 2H_2O$/bag (0 or 30 kg Mo/ha).

Twenty-four replicates were established for each of the twenty-eight treatments; twelve were shaded and twelve left unshaded. Shading was achieved by covering the soil surface with pieces of jute supported above the soil surface by small sticks. Maximum light intensity in a coffee plantation around a coffee trunk was 47 400 ± 1 400 S.E. lux. Maximum light intensity under a piece of jute was 21 900 ± 1 800 lux, where as for unshaded soil a maximum light intensity of 81 700 ± 5 700 lux was measured. Maximum temperatures of shaded and unshaded soil were 20.9 ± 0.7° C and 23.9 ± 0.6° C, respectively. Mean relative humidity between 6:00 a.m. and 6:00 p.m. as measured just above the soil

surface of unshaded bags was 74.4 ± 3.5 (highest value of 100 and lowest value of 61.0). For shaded bags under a piece of jute the relative humidity was 76.7 ± 3.8% (highest value of 100 and lowest value of 60.9). To assure adequate inoculum was present fresh nodules from the coffee plantation were crushed mixed with water to form a slurry and applied in equal amounts with a pipette with a pipette to each bag.

Seedlings were harvested 8, 10, 12, and 14 months after the fertilizer additions with 3, 3, 2, and 3 plants harvested on each date respectively. During the third harvest one replicate had to be discarded. Seedlings were removed from each bag with care taken to extract the entire root mass. The aerial and below ground portions of the seedlings were separated and the roots with nodules were placed in glass jars and assayed for acetylene reduction activity[9].

Following the assay nodule number and fresh and dry weights as well as root, stem, and leaf dry weights were determined. The soil from each bag was carefully examined for nodules that might have fallen off the roots during removal of the plants from the bag. If found these nodules were included in the nodule number and biomass calculations but not assayed for N fixing activity. Dry weights were obtained after drying materials at 80° C for 48 hs.

When appropriate analysis of variance and Duncan's multiple range test were carried out using a computer.

Results and discussion

Appearance of nodules and acetylene reduction activity

Only 30% of the seedlings harvested at 8 months were nodulated and none of these reduced acetylene (Table 1). However, by 12 months the majority of plants had nitrogen fixing nodules. The initial lag in nodule production and activity may be related to the availability of nitrogen in the soil (0.273% total N) and or the nitrogen reserves of the large *I. jinicuil* seed which average about 500 mg dry weight and has a nitrogen content of 3.92%.

Nodule number and fertilizers

Of all nutrients tested only phosphorus and nitrogen significantly affected nodule production. Phosphorus had its greatest effect on nodule number during the first 10 months of growth (Table 2). Plants grown with intermediate phosphorus levels produced significantly more nodules than either controls or

Table 1. Nodulation and acetylene reduction vs seedling age.

Seedling age (months)	Number of seedlings	Percent nodulated	Percent of nodulated plants with C_2H_2 reduction activity
8	166	30	0
10	162	47	40
12	107	76	70
14	158	95	97

Table 2 Nodule number as superphosphate*

Superphosphate (g per bag)	Seedling age (months)			
	8 n = 40	10 n = 40	12 n = 27	14 n = 39
0	0 5 a**	1 9 a	22 0 a	171 5 a
2 1	0 1 a	6 6 b	33 5 a	236 5 a
5 3	3 1 b	7 1 b	23 5 a	211 0 a
10 5	2 3 ab	4 0 ab	25 8 a	244 6 a

* Values presented are the mean number of nodules plant date

** Means followed by the same letter for the same age are not significantly different at the $p \leq 0.05$ level

those seedlings given the highest phosphorus amendment However by month 12 these differences disappeared The fact that high phosphorus fertilization did not enhance nodulation over lesser amounts agrees with the findings of Dementerio *et al*[4] who showed that excess phosphorus decreased nodule production in two varieties of soybeans

Neither potassium, magnesium, nor molybdenum significantly affected nodule number (Table 3)

Table 3 Nodule number as nitrogen potassium magnesium and molybdenum*

Treatment (per bag)	Seedling age (months)			
	8 n = 24	10 n = 23	12 n = 15	14 n = 23
0	1 2 a**	7 6 a	26 4 ab	267 0 a
0 92 g urea	0 3 a	0 2 b	5 3 bc	118 5 bc
4 6 g urea	0 a	0 b	0 2 c	24 2 c
0 4 g KCl	2 0 a	7 6 a	30 3 ab	267 0 a
2 0 g KCl	2 2 a	4 5 ab	47 4 a	282 9 ab
2 7 g MgO + 2 3 g $MgCl_2 \cdot 6H_2O$	2 0 a	1 9 ab	45 4 ab	242 1 a
0 3 g $Na_2MoO_4 \cdot 2H_2O$	4 0 a	5 6 ab	28 2 ab	246 1 a

* Values presented are the mean number of nodules plant date

** Means followed by the same letter for the same age are not significantly different at the $p \leq 0.05$ level

Nodule biomass and fertilizers

In general plants grown with additional phosphorus produced a higher nodule biomass than controls (Table 4) In contrast to nodule number highest biomass at 8 months occurred on plants grown with highest phosphorus level However with time maximum nodule biomass was associated with lower phosphorus levels These results suggest that high phosphorus levels only favour nodulation initially Overall moderate amounts of phosphorus fertilizer yielded highest nodule biomass

Nitrogen fertilization led to decreased nodule biomass compared to controls (Table 5)

Plants grown with the high level of potassium produced significantly less nodule biomass than control seedlings This difference was most pronounced for older plants (Table 5) Several studies have previously shown that potassium sulphate additions led to increased nodule biomass[5,14] However since sulfur can positively affect nodulation[10] the response reported in the above studies may have been due to the added sulfur

Neither magnesium nor molybdenum affected nodule biomass (Table 5)

Nitrogen fixation

Acetylene reduction activity was first detected on 10 month-old seedlings (Table 1) Seedlings receiving 5 3 g P exhibited significantly higher acetylene reduction activity at 10 and 12 months than plants given other phosphorus levels (Fig 1) However by the final harvest the acetylene reduction activity for all phosphorus treatments and the controls were similar These data indicate that the nitrogen fixation response of *I jinicuil* seedlings to added phosphorus is not

Table 4 Dry weight (mg*) nodules seedling vs phosphorus level

Superphosphate (g per bag)	Seedling age (months)			
	8 n = 40	10 n = 40	12 n = 27	14 n = 39
0	0 2 a**	8 7 a	32 6 a	628 3 a
2 1	0 0 a	9 1 a	128 1 b	677 3 ab
5	1 3 a	7 4 a	75 2 a	804 7 b
10 5	5 6 b	3 1 a	59 4 a	730 0 ab

* Values shown are means weight in mg seedling date

** Means followed by the same letter for the same age are not significantly different at the $p \leq 0.05$ level

Table 5. Dry weight (mg*) nodules seedling vs fertilizers

Treatment (per bag)	Seedling age (months)			
	8 n = 24	10 n = 23	12 n = 15	14 n = 23
0	3.3 a**	13.2 a	95.0 ab	866.2 a
0.92 g urea	0.1 a	0.1 a	5.2 bc	38.7 b
4.6 g urea	0 a	0 a	0.1 c	7.1 d
0.4 g KCl	1.3 a	4.5 a	101.2 a	959.5 a
2.0 g KCl	1.2 a	3.8 a	74.0 abc	556.0 bc
2.7 g $MgO + 2.3$ g $MgCl_2 \cdot 6H_2O$	2.0 a	2.4 a	97.4 ab	822.5 ab
0.3 g $Na_2MoO_4 \cdot 2H_2O$	0.3 a	6.5 a	69.5 abc	811.5 ab

* Values shown are mean weight seedling date

** Means followed by the same letter for the same age are not significantly different at the $p < 0.05$ level

linear. We found, as did Dementerio *et al*.[4] for herbaceous legumes and Gibson[7] for bacteroids, that high levels of phosphorus can inhibit nitrogen-fixing activity. The results also suggest that the amount of phosphorus applied during early plant growth can affect the rate at which nitrogen-fixing activity develops. Seedlings that received 5.3 g of superphosphate not only exhibited the highest activity of all phosphorus treatments but also achieved maximum rate of fixation two months earlier than plants which had received more or less phosphorus. If higher nitrogen-fixing activity during early growth aids plant establishment and survival, then knowing the amount of phosphorus to apply, and when, can be of practical importance. Particularly since with time plants grown with different levels of phosphorus all exhibited similar nitrogen fixing activity. The lack of differences between phosphorus treatments at 14 months may reflect the high phosphorus-fixing ability of soils containing allophanic clays.

The inhibition of nitrogen-fixing activity by fixed nitrogen compounds has been well documented for herbaceous legumes[11,12,15]. We found that nitrogen fixation by *I. punctata* seedlings was also depressed in the presence of nitrogen fertilizer (Table 6).

Low level potassium fertilization significantly increased acetylene reduction above control values for the first ten months (Table 6). However, high levels of potassium depressed activity. Mengel *et al*.[14] and deMooy and Pesek[3] had also found that potassium stimulated nitrogen fixation by *Vicia faba* and *Glycine max*, respectively. However, neither of the above studies showed that high levels of potassium were inhibitory to nitrogen fixing activity. In both these studies[3,14]

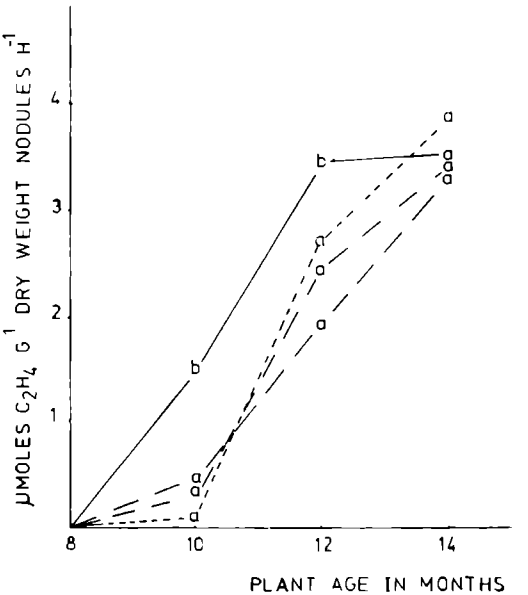

Fig. 1 Acetylene reduction activity of *I. jinicuil* seedlings is affected by different phosphorus gifts Control 2.1 g superphosphate per bag 5.3 g superphosphate per bag 10.5 g superphosphate per bag Values interrupted by the same letter for the same age are not significantly different at the $p \leq 0.05$ level

potassium sulphate had been applied while in our study potassium chloride was used High levels of chlorine have been shown to negatively affect biomass accumulation in herbaceous legumes We obtained similar results with *I. jinicuil* seedlings reduced biomass of seedlings grown with high potassium chloride as compared to control plants Since chlorine affects biomass accumulation it may also influence nitrogen fixing activity

Although we previously reported a positive correlation between magnesium and nitrogen fixation [1] plants receiving magnesium fertilizer in this study did not have significantly higher acetylene reducing activity than control plants (Table 6) Again chlorine added with magnesium may have depressed nitrogen fixing activity Finally the acetylene activity of plants grown with increased molybdenum was not significantly different from controls (Table 6)

Soil shading

The soil surface in many coffee plantations received little direct sunlight

Table 6 Acetylene reduction activity* vs fertilizers

Treatment (per bag)	Seedling age (months)		
	10 n = 23	12 n = 15	14 n = 23
0	986 a**	3075 bc	4034 a
0.92 g urea	0 b	605 d	4050 a
4.6 g urea	no nodules	no nodules	1975 b
0.4 g KCl	813 b	4947 a	3493 a
2.0 g KCl	309 ab	1411 d	3141 a
2.7 g MgO + 2.3 g $MgCl_2 \cdot 6H_2O$	470 ab	3071 bc	3243 a
0.3 g $Na_2MoO_4 \cdot 2H_2O$	825 a	3794 ab	3763 a

* Values presented are the mean nmol C_2H_4 reduced per 1 g dry weight nodule h

** Means followed by the same letter for the same age are not significantly different at the $p \leq 0.05$ level

because of shade from coffee and shade plants A recent trend in coffee cultivation in Mexico calls for the elimination of shade trees to reduce fungal disease and accelerate coffee yields We were therefore interested in what effects if any shade had on nodulation and nitrogen fixation

We found that irrespective of fertilizer treatment plants grown in bags whose soil was unshaded produced significantly more nodules and a larger nodule biomass throughout most of the study (Fig 2) These differences are most likely due to temperature and moisture effect of shading

Xalapa has warm summers and cool winters with high relative humidity and abundant rainfall Soil in bags without shade could be warmer on sunny days Higher soil temperatures could result in increased evaporation and thereby improve soil aeration At the same time increased soil temperatures would stimulate microbial activity More oxygen in the root zone and increased microbial activity could lead to higher nodule number and biomass

The same explanation may also apply to acetylene reduction activity which was higher for unshaded bags during the warm summer months Several reports[11,13] have recently demonstrated the large effect soil temperature can have on nitrogen fixing activity With the onset of winter and cooler temperatures in November maximum activity was found in shaded bags The data suggest that as ambient temperatures drop shaded soil may maintain higher temperatures and therefore support higher activity Nodule biomass for November may be higher in unshaded bags because biomass is accumulated through time and

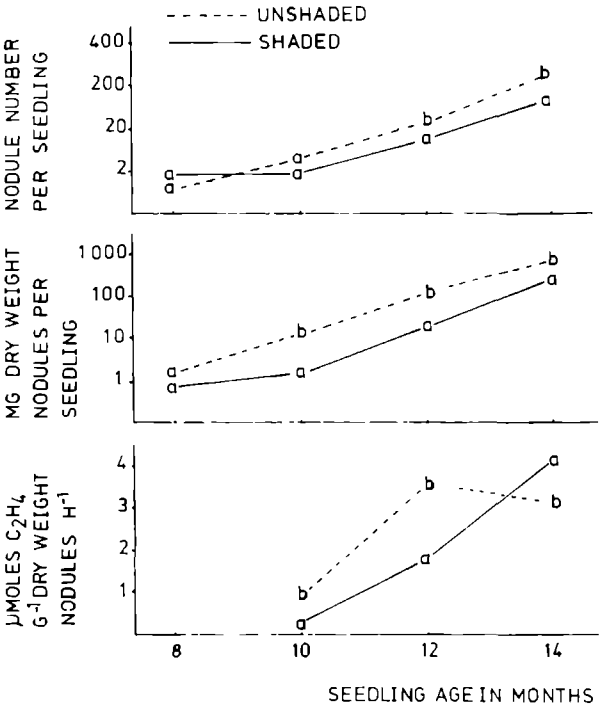

Fig 2 Effect of soil shading vs non shading on nodule number biomass and acetylene reduction activity of *I jinicuil* seedlings Values interrupted by the same letter for the same age are not significantly different at the $p \leq 0.05$ level

reflects prior environmental conditions, while acetylene-reduction activity is a function of conditions existing immediately before assay

These results indicate that nodulation and nitrogen fixation by *I jinicuil* in densely shaded plantations may not be optimal during summer months However, the occurrence of high levels of activity during cool winter months because of the insulating effect of shade may more than compensate for reduced summer activity In any event, it appears that shade tree density may not only affect coffee production directly but indirectly, as well, by influencing nitrogen fixation It should be noted that the data presented here are for seedlings Whether adult *I jinicuil* trees behave similarly has yet to be determined but seems likely given the ease of finding nodules in open, sunny spots in the coffee plantation but not in heavily shaded ones

General discussion

Of all the treatments tested phosphorus nitrogen and shading appear to have the greatest effects on nodulation and nitrogen fixation by *I. punctata* seedlings

The response of *I. punctata* to phosphorus fertilization was not uniform High phosphorus additions led to high nodule biomass in young seedlings while maximum nitrogen fixing activity in those same plants occurred when medium levels of phosphorus were applied By the time the seedlings were one year old nodule number and activity were similar for all phosphorus treatments and not significantly different from controls Thus the positive effects of phosphorus appear most pronounced on the youngest plant Overall the results suggest that nodulation and N_2-fixation by *I. punctata* are increased when phosphorus is applied This evidence substantiates findings of an earlier study we hypothesized that nodulation and phosphorus were related[1] Finally from a practical point of view it appears that more phosphorus will not necessarily produce more fixation even in phosphorus fixing tropical soils

Nitrogen fertilization inhibited nodulation and nitrogen fixation throughout the experimental period Given the bulk of studies on herbaceous legumes where similar findings have been obtained our result was not unexpected However one question needs yet to be resolved If nitrogen is as inhibitory to nodulation and nitrogen fixation as our results indicate how is it possible for adult *I. punctata* trees to nodulate and fix nitrogen in coffee plantation where the soil nitrogen content is over 0 5% total Kjeldahl nitrogen?

Soil shading significantly decreased nodule number and biomass throughout the experiment Similarly during the summer months shaded plants reduced less acetylene However this pattern reversed at the beginning of the winter season High activity was then associated with plants grown in shaded bags We believe these differences are due to the effect that shading has on soil moisture and temperature Coffee growers who wish to optimize production may now have to consider the effect of shade on shade trees as well as on coffee plants

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CHAPTER 3.

$^{15}N_2$-fixation and H_2 evolution by leguminous trees.

ABSTRACT

The $C_2H_4/^{15}N_2$ and $H_2/^{15}N_2$ ratios for six species of tropical leguminous trees are reported. $C_2H_4/^{15}N_2$ ratios are ranged from 2.4 to 4.7; values for the $H_2/^{15}N_2$ ratios were between 0.6 and 1.4. Relative efficiency values, based on C_2H_2 reductions, ^{15}N-incorporation, and H_2 evolution during ^{15}N-incorporation varied between 0.68 and 0.84 for the six species. Overall, approximately 30% of the electron flow through nitrogenase was used for H_2 evolution.

INTRODUCTION

Recently, interest in leguminous trees has increased (9, 10, 11, 12). The value of these trees for forage, timber, firewood, in land reclamation and against soil erosion has long been underestimated. Furthermore, many leguminous trees are nodulated (1), and can therefore increase the total amount of nitrogen present in tropical agro-ecosystems through N_2-fixation.

In most studies, the N_2-fixing activity of leguminous trees is measured by the acetylene reduction technique (6, 7, 8, 15, 17). Because acetylene is not the physiological substrate for nitrogenase, mol C_2H_2 reduced is converted to mol N_2 fixed especially when an estimate for the nitrogen input into ecosystems from fixation is desired.

As Burris (3) pointed out, the theoretical ratio of C_2H_4/N_2 of 3 is rarely observed experimentally and an empirical ratio, based on $^{15}N_2$ reduction under specific experimental conditions, should be established. One reason for the discrepancy between the theoretical and empirical is the production of H_2 by nitrogenase during the N_2-fixation (5, 14). However, by measuring H_2 evolution under atmospheric conditions and during acetylene reduction,

the relative efficiency of nitrogenase can be determined (16).

In this communication we report and compare $C_2H_4/^{15}N_2$ ratios, H_2 evolution and relative efficiency, based on acetylene reduction and ^{15}N-incorporation for six species of tropical leguminous trees.

MATERIALS AND METHODS

Plant material

Seeds of Acacia pennatula (Cham. and Schlecht.) Benth., Albizia lebbeck (L.) Benth., Enterolobium cyclocarpum Griseb, Gliricidia sepium (Jacq.) Steud, and Leucaeana leucocephala (Lam.) de Wit (= glauca (L.) Benth.) were planted in May 1982 in plastic bags, containing approximately 7 kg. of topsoil, collected from pastures at Uxpanapa, Vera Cruz, Mexico. Seeds of Inga jinicuil Schlechter were also planted in plastic bags, containing seven kg. of soil, collected from the Botanical Garden of Xalapa, Vera Cruz, Mexico in October 1979. Bags of the first five species were placed at the INIREB (Instituto Nacional de Investigaciones sobre Recursos Bioticos) experimental field station Morro de la Mancha, located at sea level near the city of Vera Cruz, Mexico. Bags with seeds of I. jinicuil were placed at the Botancial Garden in Xalapa.

Assays

Four-and-a-half months after planting, plants were removed from the bags and nodules separated from the roots. Several nodules were used for acetylene reduction and the remaining nodules from the same plant were subjected to ^{15}N-incorporation. To avoid transport of fixed ^{15}N into roots, only excised nodules were used in the assays. Acetylene reduction was initiated by adding 10% by volume acetylene, generated from CaC_2, to incubation vials. After an

incubation of 60 min., gas samples from the vials were transferred to 5 ml. vacutainers and later analyzed for C_2H_4 and H_2 at the Department of Biochemistry, Madison, Wisconsin. C_2H_4 was measured by gas chromatography with an Aerograph 600 D unit, equipped with a flame ionization detector; N_2 was the carrier gas. H_2 was measured gas chromatographically with a Gow-Mac Series 150 unit equipped with dual columns and a thermal conductivity detector. Argon was used as the carrier and the column temperature was 75°C.

$^{15}N_2$-fixation assays were performed at the same time as the C_2H_2 reduction tests, under the same incubation conditions. Incubation vials were sealed with vaccine stoppers, and then evacuated through a manifold with a rotary vane vacuum pump. After evacuation for about 10 sec., a gas mixture of 20% O_2, 30% $^{15}N_2$, and 50% argon was added to each vial. ^{15}N-enriched $(NH_4)_2SO_4$ was converted to $^{15}N_2$ with NaOBr. The enriched $^{15}N_2$ gas was first mixed with alkaline $KMnO_4$ and subsequently with 6 N H_2SO_4 to remove any nitrogen oxides and ammonia (3); it was then added to the incubation vials containing the excised nodules. After incubation for 60 min., gas samples from the vials were transferred to 3 ml. vacutainers. The atom percent ^{15}N of the stored gas mixtures was determined with a MAT 250 isotope ratio mass spectrometer. Because of the possible nonequilibrium state of masses 28 ($^{14}N^{14}N$), 29 ($^{14}N^{15}N$), and 30 ($^{15}N^{15}N$), all three peaks were measured (3). H_2 was measured gas chromatographically as mentioned before. Unused vacutainers were checked for residual gases.

Nodules were dried for 48 hours at 70°C, weighed, and transferred to micro-Kjeldahl flasks. After digestion with H_2SO_4, and $HgCl_2$ as a catalyst, powered Zn was added to amalgamate the Hg. The solution was made alkaline and a steam distilled for 7.5 min. Samples were distilled into 10 ml. of 0.036 N H_2SO_4. Ammonium concentrations of the distillates were determined with Nessler's reagent (4). Nodules used for

the acetylene reduction assays, digested the same way, were used to determine the natural abundance of ^{15}N. NaOBr was used to convert ammonium to N_2. The R value of the samples (R = M29/M28) was calculated by a Hewlett Packard 9815-A minicomputer connected to the mass spectrometer. Atom percent ^{15}N was calculated by the formula: Atom percent ^{15}N = 100R/(2+R). Atom percent ^{15}N due to fixation equaled the difference in atom percent ^{15}N of the nodules exposed to $^{15}N_2$ and the atom percent ^{15}N of the nodules used for the acetylene reduction assays.

RESULTS AND DISCUSSION

Rates of C_2H_4 production, H_2 evolution during ^{15}N-incorporation, and $^{15}N_2$-fixation of the species are given in Table 1. According to Allen and Allen (1), Rhizobium able to nodulate tropical trees, belong to the Cowpea group, which can evolve small amounts of H_2 under atmospheric conditions (16). However, all the symbionts tested in this experiment evolved substantial amounts of H_2 during $^{15}N_2$-fixation. At the same time, no H_2 evolution was detected during C_2H_2 reduction assays. Five of the six species examined yielded $H_2/^{15}N_2$ ratios greater than 1.

Table 1 shows the $C_2H_4/^{15}N_2$ and $H_2/^{15}N_2$ ratios and the relative efficiencies of the symbionts. To our knowledge, no C_2H_4/N_2 and $H_2/^{15}N_2$ ratios for leguminous trees have been reported and therefore comparison with similar experiments is not possible. Relative efficiency is usually defined as follows (13):

$$\text{Relative Efficiency (RE)} = 1 - \frac{\text{rate of } H_2 \text{ production in air}}{\text{rate of } C_2H_4 \text{ production}}$$

In addition the R.E. of the symbiont can be measured using the physiological substrate, N_2, and is then defined by the following equation:

Table 1: C_2H_4 production, $^{15}N_2$-fixation, H_2 evolution, $C_2H_4/^{15}N_2$, and $H_2/^{15}N_2$ ratios, and R.E (Relative Efficiency) for six species of leguminous trees.

Plant Species	Rep n	C_2H_4 [1]	$H_2(^{15}N_2)$	$^{15}N_2$	$\frac{C_2H_4}{^{15}N_2}$	$\frac{H_2}{^{15}N_2}$	R.E. [2] $(^{15}N_2)$	R.E. [3] (C_2H_4)
Acacia pennatula	13	13.1 ± 2.9	4.2 ± 0.4	3.5 ± 1.0	3.7	1.2	0.71	0.68
Albizia lebbeck	11	27.3 ± 6.3	7.7 ± 1.5	5.9 ± 1.6	4.6	1.3	0.70	0.72
Enterolobium cyclocarpum	14	16.4 ± 4.9	6.1 ± 0.8	4.4 ± 0.9	3.7	1.4	0.68	0.63
Gliricidia sepium	11	14.9 ± 6.2	6.1 ± 1.0	4.5 ± 1.9	3.3	1.4	0.69	0.59
Leucaena leucocephala	10	11.4 ± 2.6	7.6 ± 1.1	5.3 ± 1.5	2.2	1.4	0.68	0.33
Inga jinicuil	21	16.4 ± 1.9	2.6 ± 0.3	4.6 ± 0.7	3.6	0.6	0.84	0.84

1 Values presented are μmoles g(dry weight)$^{-1}h^{-1}$ ± error of mean.

2 R.E. $(^{15}N_2) = 1 - \frac{\text{rate of } H_2 \text{ production in air}}{3\text{x rate of } ^{15}N_2\text{-fixation} + \text{rate of } H_2 \text{ production in air}}$

3 R.E. $(C_2H_4) = 1 - \frac{\text{rate of } H_2 \text{ production in air}}{\text{rate of } C_2H_4 \text{ production}}$

$$\text{R.E.} = 1 - \frac{\text{rate of } H_2 \text{ production in air}}{3\text{x rate of } {}^{15}N_2\text{-fixation} + \text{rate of } H_2 \text{ production in air}}$$

The R.E. for the six species, whether based on C_2H_2 reduction or on $^{15}N_2$-fixation, did not vary greatly except for the Leucaena - Rhizobium symbiont (Table 1). This symbiont yielded a $C_2H_4/^{15}N_2$ ratio lower than the theoretical value of 3. However, the $H_2/^{15}N_2$ ratio, rate of $^{15}N_2$-fixation, and R.E. based on $^{15}N_2$-fixation were within the range of the other symbionts tested. At present we are at a loss to explain the unexpectedly low $C_2H_4/^{15}N_2$ ratio for this species.

I. jinicuil had the lowest rate of H_2 evolution and the highest R.E. However, the I. jinicuil plants assayed were 2½ years older than the seedlings of the other species tested. It has been shown that during the ontogeny of Alaska pea and cowpea, H_2 evolution decreased compared to the total electron flux through nitrogenase (2) or to N_2-fixation (13). If this phenomenon holds for other legumes, it could explain the observed differences in H_2 evolution and R.E. between I. jinicuil and the other five species.

It is evident from this study that the Rhizobium - leguminous tree symbionts suffer an energy loss of about 30% during the process of N_2-fixation, due to the production of H_2. This led to relative efficiencies averaging 0.70 for the six species tested; a value greater than that reported for most legumes, i.e. 0.40 to 0.60 (16) but lower than was expected.

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CHAPTER 4

Annual, seasonal and diurnal variation in nitrogen fixing activity by *Inga jinicuil* Schl., a tropical leguminous tree.

SUMMARY

Inga jinicuil Schlechter is a leguminous tree, native to the secondary successional rainforests of Mexico, that is used as a shade tree in coffee plantations. Annual, seasonal and diurnal patterns in nitrogen-fixing activity by this species were monitored over a three-and-a-half year period using the acetylene reduction technique.

Year to year variation was unexpectedly large, suggesting that a longer sampling period was needed to accurately assess mean annual fixation.

Pronounced seasonal changes in nodular activity appear to be determined by *Inga* phenology which is controlled by variations in precipitation and temperature. Nitrogen-fixing activity occurred throughout the year but was highest in the summer and fall when precipitation and temperature were at a maximum and when the majority of tree growth and reproduction occurred. *I. jinicuil* flowered twice, annually and nodular activity peaked once during each reproductive cycle. Maximum activity occurred post-flowering in the first reproductive cycle and pre-flowering in the second.

Diurnal fluctuations in nitrogen fixation rates were obtained on most but not all sampling dates. However, the observed patterns of activity varied from date to date. Aside from an activity peak that occurred at 1900 h, averaged rates of nodular activity were remarkably constant throughout the day. Seedlings fixed 35% more nitrogen than 30-year old trees but had a similar diurnal activity pattern.

Overall, the results show that variability in nitrogen-fixing activity was large between years, pronounced but explainable between months, and relatively small between hours of the day. The timing of maximum and minimum activity, both seasonally and daily, differed significantly from what has been reported for most other nitrogen-fixing species. This suggests that studies, attempting to assess annual fixation by previously unstudied species, should sample as

intensively and extensively as possible over time to adequately encompass temporal variation.

INTRODUCTION

Leguminous trees are abundant in many primary and successional tropical forests (Forman and Hahn 1980; Knight 1980; Sylvester-Bradley et al. 1980; Rzedowski 1978) but little is known about their biology or ecology. Recently, world interest in tree legumes has increased because many are fastgrowing and can supply resources needed by developing tropical nations (Brewbaker et al. 1982; N.A.S. 1980).

Woody legumes can provide high-protein forage and fodder, nitrogen-rich green manure, fuel, timber, other wood products, and help control soil erosion (Roskoski et al. 1981; Brewbaker et al. 1982; N.A.S. 1977, 1979, 1980). In addition, many leguminous trees fix atmospheric nitrogen thereby increasing the nitrogen content of ecosystems in which they occur (Roskoski et al. 1982; N.A.S. 1977, 1979). Despite the potential importance of nitrogen inputs to natural and agro-ecosystems in the tropics from tree legumes, many aspects of nitrogen fixation by these species are poorly understood. In particular, little data exists on temporal variations in nitrogen-fixing activity.

In 1977 the Instituto Nacional de Investigaciones sobre Recursos Bioticos in Xalapa, Veracruz, Mexico initiated nitrogen cycling studies in coffee plantations. As part of those studies, in 1979 we began an investigation to quantify annual nitrogen inputs to the coffee ecosystem from nitrogen fixation by a leguminous shade tree, Inga jinicuil Schlechter. Quantification of annual fixation required data on both nodular biomass and activity. Since most studies on nitrogen fixation measured over time have encountered temporal variability, we ran a series of studies to assess annual, seasonal, and diurnal variation in nitrogen-fixing activity by I. jinicuil. This paper presents the results of those studies.

MATERIALS AND METHODS

The study took place in and near Xalapa, Veracruz, Mexico; 19°27' N, 96°57' W, 1225 m elevation. The climate of the area is classified as semi-hot humid, with warm summers and cool winters (Garcia 1970). Annual mean temperature is 19°(± 2°) C, and annual precipitation averages 1758 (± 193) mm. The soil is an inceptisol, suborder=andept, derived from volcanic ash, with a high content of phosphorus-fixing allophanic clays (Ramos et al. 1982).

I. jinicuil is not native to the Xalapa area. It naturally occurs in secondary vegetation derived from perennial tropical forests (Rzedowski 1978), and was introduced into the Xalapa region around 1900 as a shade tree for coffee plantations.

Nodules were collected from a coffee plantation containing I. jinicuil shade trees (205/ha), coffee plants (1600/ha), and sparse ground cover dominated by Commelina spp. (Jimenez-Avila 1979). The Inga trees were 14-16 meters in height and had a mean DBH (diameter at 1.5 m) of 33.9 ± 1.48 $s-_{x}$ cm. Growth ring analysis indicated that the trees were approximately 30 years old.

Once a month from March 1979 through April 1982, 10 nodule samples were randomly collected at 0700, 1000, 1300, and 1600 h and assayed for nitrogen-fixing activity using the acetylene reduction technique (Hardy et al. 1973). Care was taken to include at least 2 cm of root containing the nodule sampled. Incubation period for the acetylene reduction assay was 1 hour. Since nodules were concentrated around coffee trunks within or slightly below the litter layer (Roskoski 1981), it was relatively easy to locate nodules without seriously disturbing either coffee or Inga roots. In addition to the sampling scheme just described, from October 1979 through October 1980, 10 nodule samples were also collected at 1900, 2300, 0300, 0700, 1000, 1300, and 1600 h and assayed for nitrogen-fixing activity. At each sampling time on each

day soil temperature at a depth of 5 cm was measured. In addition, the phenological state of the trees was recorded on each sampling date. Moles C_2H_2 reduced was converted to moles N_2 fixed using an empirically determined ratio of 3.6:1 (Van Kessel et al. in press). Following assay, nodules were separated from the attached root segment, weighed fresh, oven-dried for 48 h at 80°C, and re-weighed.

In September 1980, nodules from one-year-old seedlings, which had been grown in plastic bags containing soil from the same area as the coffee plantation, were assayed for nitrogen-fixing activity at the same times as the adult trees. Shoots were removed from 5 seedlings/ sampling time and the roots + nodules carefully extracted from the soil. Two nodule samples were selected from each root mass and individually assayed for nitrogen-fixing activity. The remaining root mass and nodules were also assayed. After assay, nodules were dried and weighed as described above. In addition, the roots, stems, and leaves from each seedling were oven-dried and weighed.

Statistical treatment of the data was done with SPSS (Statistical Programs for Social Sciences) package of programs and a CYBER 175 computer.

RESULTS AND DISCUSSION

Annual Variation in N_2-Fixation

Data for 0700, 1000, 1300, and 1600 h from June through October 1979- 1981 were analyzed for differences in annual nitrogen-fixing activity. Only data for June through October were used because assays had been performed in each of these months in each year and these five months accounted for 60%, 71%, and 59% of the total annual fixation in 1979, 1980, and 1981, respectively.

Analysis of variance revealed that the three annual means were significantly different (F of 38.10, df= 2/817, p= 0.0001). Duncan's multiple range test furthermore showed that

each annual mean was significantly different from every other mean (Table 1). The highest annual rate was found in 1980 and the lowest in 1979.

No other studies have examined annual variations in nitrogen fixation rates by woody perennial legumes. But Whiteman and Lulham (1970a) did document annual changes in nodule biomass for two herbaceous perennial species. At an age of thirty years, the Inga trees are reproductively mature and probably maintain relatively constant leaf biomass from year to year. Thus the marked yearly variation we observed was quite unexpected. Two phenomena which began in 1980 may be responsible for the increased yearly activity seen in 1980 and 1981.

Until 1980 the study area had been heavily fertilized with N-P-K or urea at a rate of 1600 kg N/ha/yr. Starting in 1980 no fertilizer was applied. Since I. jinicuil only fixed 20% of its annual nitrogen demand when fertilizers were being used, the withdrawl of fertilizer nitrogen may have promoted an increase in nitrogen fixing activity. At the same time nodulation and nitrogen fixation by I. jinicuil are strongly inhibited by fixed nitrogen compounds (Van Kessel and Roskoski 1981; Van Kessel and Roskoski 1983). Cessation of fertilization would lead to a decreased level of fixed nitrogen in the soil thereby reducing inhibition of nitrogen fixation.

A second factor that may have been responsible for the increase in yearly activity was a severe insect defoliation which occurred in June and July 1980. As a result of this perturbation all foliage was stripped from the trees and the developing, immature pods abscissed. Evidence from other species showed that following defoliation nodule biomass first drops (Butler et al. 1959; Whiteman and Lulham 1970b; Bowen 1959; Igwilo 1981) and then rises as a flush of new nodules occurs (Butler et al. 1959; Whiteman and Lulham 1970b). Apparently, the new nodules may be more active than the pre-defoliation ones (Igwilo 1981). In addition to

Table 1. Annual Nitrogen Fixation.

Year	u Moles N_2 Fixed g^{-1} Nodules h^{-1} (± SE) June	July	August	September	October	n	x of June-Oct.	
1979	0.96 (0.13)	3.84 (0.41)	2.52 (0.32)	2.42 (0.23)	2.13 (0.20)	233	2.36 (0.13)	a[1]
1980	4.47 (0.34)	4.91 (0.39)	1.66 (0.18)	3.83 (0.28)	7.63 (0.49)	391	4.50 (0.18)	b
1981	2.73 (0.21)	4.53 (0.46)	3.79 (0.30)	3.21 (0.36)	4.44 (0.37)	196	3.74 (0.17)	c

[1] means with different letters are significantly different, p = 0.05.

defoliation, depodding can stimulate nitrogen-fixing activity. Several studies have established that by removing a competing photosynthetic sink, depodding results in increased nitrogen fixing activity (Lawrie and Wheeler 1974; Young 1981; Ham et al. 1976). Thus the combined effects of cessation of fertilization, defoliation, and depodding may be responsible for the high annual rate of N_2-fixation observed first in 1980.

Seasonal Variation in N_2-Fixation

Monthly means, based on data from all years and hours 0700 through 1600, were significantly different (F of 24.91, df= 11/1292, p= 0.0001). Highest monthly activity occurred in October and lowest monthly rates were in January and April (Table 2). In general, activity was high in the summer and fall (June through October) and low in the winter and spring (November through April). Duncan's multiple range test indicated that three significantly different groups of means existed. Group 1 was composed of months July and October, group 2 contained means for June and September, and group 3 contained all other means (Table 2).

Figure 1 presents plots of mean monthly temperature, precipitation, nitrogen-fixing activity, and the observed phenology of I. juniculi. April is shown as the first month on the graphs since this is when flowering begins, thus starting the phenological year. Air and soil temperatures on any one date are similar and will be referred to collectively as temperature in the following discussion.

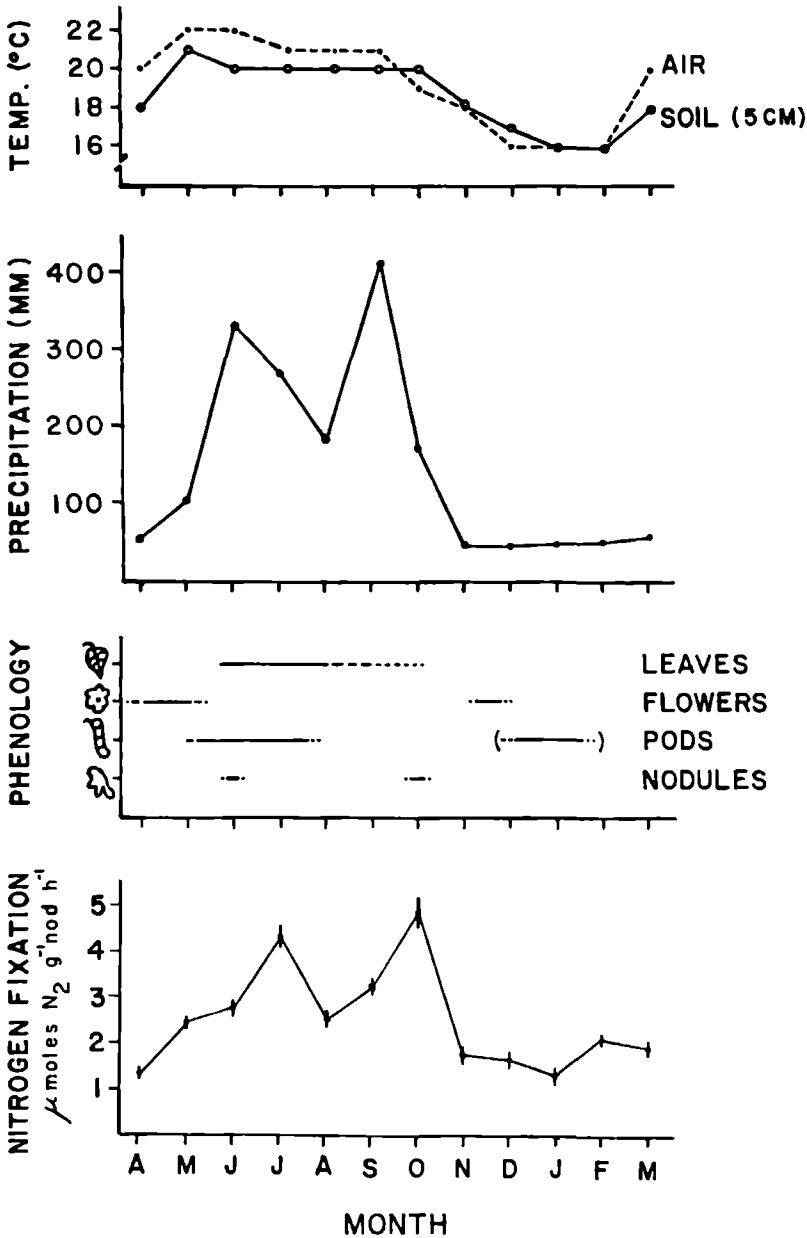
When flowering begins in April, temperature approximates the yearly mean of 19°C, but precipitation and nodular activity are at their yearly minimum. As flowering continues in May, temperatures rise to a yearly maximum, and precipitation and nitrogen fixing activity begin to increase. Temperature falls slightly in June as the summer rains begin. During this period nodular activity continues to rise and several important phenological events coincide. Pod

Table 2. Monthly nitrogen fixing activity.

Months	n	μ Moles N_2 Fixed g^{-1} Nodules h^{-1}
Jan.	75	1.54 ± 0.16 a[1]
Feb.	101	2.15 ± 0.18 abcd
Mar.	151	2.01 ± 0.12 abc
April	145	1.54 ± 0.13 a
May	79	2.41 ± 0.23 bcd
June	121	2.76 ± 0.24 de
July	121	4.38 ± 0.26 f
Aug.	112	2.58 ± 0.18 cd
Sept.	120	3.25 ± 0.20 e
Oct.	121	4.92 ± 0.38 f
Nov.	79	2.17 ± 0.23 abcd
Dec.	69	1.78 ± 0.17 ab

[1]Means with a letter in common are not significantly different at p=0.05.

Figure 1. Temperature (a) precipitation (b), Inga phenology (c), and nitrogen fixing activity (d) vs. months April to March. Data for air temperature and precipitation are from Jiminez (1979). Phenological data are as follows: heavy lines represent maximum activity, dotted lines signify less activity. Lines for leaves and flowers show the months when leaf and flower production occur. The lines for pods indicate the time of pod development, and the line for nodules indicate the time of the year when new pink nodules were observed in the field. Figure 1d plots mean nitrogen fixing activity for each month ± 1 $S\text{-}_x$.


TEMP. (°C)
22
20
18
16
AIR
SOIL (5 CM)
PRECIPITATION (MM)
400
300
200
100
PHENOLOGY
LEAVES
FLOWERS
PODS
NODULES
NITROGEN FIXATION
μ moles N_2 g^{-1} nod h^{-1}
5
4
3
2
1
A M J J A S O N D J F M
MONTH

development begin, new leaves flush out, and an abundance of new pink nodules are observed in the field. From June through October temperatures remain relatively constant. In July precipitation falls slightly from June levels but nitrogen fixing activity reaches one of its two yearly maxima. New leaves are still being produced and pod filling continues. Pods begin to fall in August concomitant with a marked decrease in precipitation and nodular N_2-fixation.

The five month period (April through August), just discussed, could be characterized as a time of peak biomass production when nitrogen demands for developing vegetative (leaves) and reproductive (flowers and pods) structures is undoubtedly high. Interestingly, the highest nodular activity does not occur at the beginning of this period but near the end when nitrogen demand is probably decreasing. The late peak in activity may be because leaf biomass, which produces the photosynthate vital for nitrogen fixation, does not reach its maximum until after flowering and well into pod-filling (Jimenez-Avila and Martinez-Vara 1979).

In September precipitation increases markedly and nitrogen-fixing activity rises from its August level. During the same time the last of the pods are falling but otherwise there is little phenological activity. A pulse of leaf fall occurs in October probably prompted by a drop in precipitation. At the same time, however, a second peak in nitrogen-fixing activity occurs and new pink nodules are again abundant. Winter commences in November with a fall in temperature and precipitation. Curiously, a second flowering also occurs in November.

In each year of the study flowers were observed in November but only in 1981 were pods produced after this winter flowering. Since no pods were produced in August 1980 because of a severe insect defoliation (see Annual Variation in N_2 Fixation), the pod production in February 1981 may have been in response to this perturbation. These data suggest that while flowering occurs twice each year, pods are

produced once, preferably in the summer, when environmental conditions are more favorable for seed survival. I. juniculi seeds are viviparous and require high moisture levels for establishment. Little precipitation coupled with low temperatures may explain why no seedlings became established from the February pods. Unfavorable environmental conditions may also explain why pod production in February was visibly less than in August.

The winter months are characterized by low temperatures, precipitation, and nodular activity. Aside from the February pod production, mentioned above, little phenological activity occurs. A second pulse of leaf fall occurs in March probably prompted by low precipitation and rising temperatures. The fact that evapotranspiration exceeds precipitation in March tends to support the above hypothesis (Jimenez-Avila and Goldberg 1982).

Seasonal changes in nodular activity closely mirror seasonal variation in precipitation and to a lesser degree temperature. The effects of these environmental variables probably influence nitrogen fixation via the phenology of I. juniculi. Similar relationships between moisture, temperature and nitrogen fixation have been documented for trees (Hingston et al. 1982; Tripp et al. 1979; Högberg and Kvarnström 1982), shrubs (Schwintzer et al. 1982), and herbs (Whiteman and Lulham 1970a; Whiteman 1970b, 1970c).

Examination of nodular activity associated with each flowering-fruiting period suggests that two different patterns may occur. During the first reproductive period (April through August), maximum activity occurs after flowering and during pod-filling. This pattern, though not extremely common, has been observed for both annual (Mague and Burris 1972; Hardy et al. 1968; Igwilo 1981; Ham et al. 1976), and perennial (Bowen 1959; Whitemen 1970c) legumes. Nitrogen-fixing activity associated with the second reproductive cycle (October through February) is highest prior to flowering and low during pod-filling. This is the pattern

most commonly encountered in seasonal studies on nitrogen fixation (Sprent 1976; Bond 1936; Lawrie and Wheeler 1973, 1974; Young 1981; Weber et al. 1976). I. jamicuil seems to be unique in that it possesses both patterns. However, whether the observed two patterns are both genetically determined or are caused by environmental conditions significantly different from those of the natural habitat of I. jamicuil, is unknown.

Diurnal Variation in N_2-Fixation: Adult Trees

The data for all assays conducted between October 1979 and October 1980 were used to test for differences in hourly activity. Large variations were found in diurnal patterns. Nitrogen fixation rates were constant throughout the day on some assay dates and fluctuated dramatically on others. Never-the-less, analysis of variance revealed that hourly means were significantly different (F of 5.43, df= 6/1319, p= 0.0001). Highest nitrogen-fixing activity occurred at 1900 h, and was almost twice the lowest activity found at 1600 h; 4.34 ± 0.36 vs 2.39 ± 0.16 (Figure 2).

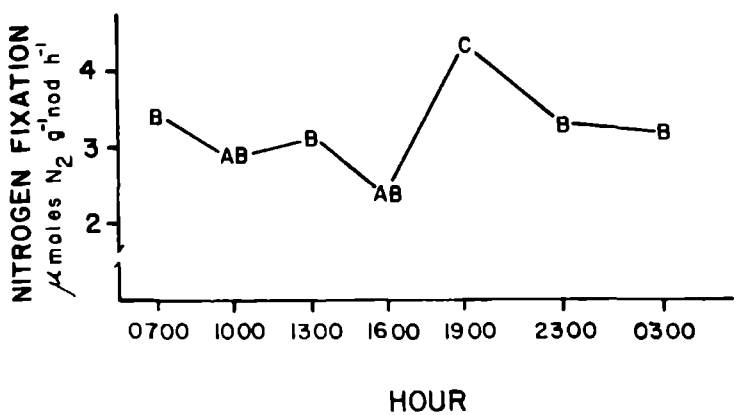


Figure 2. Nitrogen fixing activity for I. jamicuil nodules vs. hours. Letters indicate mean values, means with a letter in common are not significantly different at p= 0.05.

However, Duncan's multiple range test showed that only two significantly different groups of means existed: one group composed of the single mean for 1900 h and a second group containing all other hourly means. Two aspects of these results are noteworthy: the lack of a consistent diurnal pattern and the daily maximum occurring at 1900 h.

Most studies on diurnal nitrogen-fixing activity whether by herbaceous (Mague and Burris 1972; Hardy et al. 1976; Bergersen 1970; Greig et al. 1962; Pate 1976) or woody (Högberg and Kvarnström 1982; Langkamp et al. 1979; Wheeler 1969; Tripp et al. 1979) nitrogen-fixing species, found well-defined diurnal patterns. In contrast, Fessenden et al. (1973) encountered no diurnal pattern for Myrica gale but concluded that small sample size may have been responsible. Such an explanation in our study is unlikely since we assayed over 1300 samples during a 12 month period. Cloudy conditions are known to depress diurnal fluctuations (Lawrie and Wheeler 1976; Hardy et al. 1976). However, in the study on Inga, sunny days were just as likely as cloudy ones to have pronounced diurnal fluctuations. Consistent diurnal patterns were found for one species of alder (Tripp et al. 1979) but not for another (Akkermans et al. 1976; Wheeler and Lawrie 1976). Interestingly, 2-year-old Alnus glutinosa had no diurnal pattern but 6-month-old seedlings did (Wheeler and Lawrie 1976). Apparently, whether a consistent diurnal pattern is obtained may depend on the species studied, its age, and probably environmental conditions during the assays.

Maximum rates of nitrogen-fixing activity are usually encountered at midday when light intensity is highest (Hardy et al. 1976; Bergersen 1970; Greig et al. 1962; Höberg and Kvarnström 1982; Wheeler 1969; Tripp et al. 1979; Langkamp et al. 1979). However, a few studies have found, as we did with Inga, highest activity in the late afternoon (Mague and Burris 1972) or early evening (Wheeler and Lawrie 1976). Although the authors of the above studies attributed afternoon maxima to more favorable temperatures than at midday,

such an explanation seems unlikely for I. juniculi. The difference between the daily minimum and maximum temperatures rarely exceeded 3°C in our study. A more likely explanation may be related to the growth periodicity of trees.

Shoot elongation and probably diameter growth of trees occur primarily at night (Kramer and Kozlowski 1962). Nitrogen needed for this growth may promote high nodular activity at 1900 h.

Diurnal Variation in N_2-Fixation: Seedlings vs Trees

Nitrogen-fixing activity for nodules from 1-year-old seedlings and 30-year-old trees was found to be significantly different (t of 3.70, df= 210, p= 0.0001). Activity of seedlings was 35% higher than for trees; 6.26 vs 4.60 u moles N_2 fixed/g nodules/hour. Similar findings have been reported for alder (Wheeler and Lawrie 1976) and Leucaena leucocephala (Högberg and Kvarnström 1982).

While mean rates of activity varied between seedlings and trees, the diurnal patterns for both were similar (Figure 3). On day 1, which was sunny, a drop in activity occurred at midday. This was probably due to a midday decrease in photosynthesis, which is characteristic for many tree species (Kramer and Kozlowski 1979). Nodular activity then rose to a peak at 1900 h, fell at 2300, and rose again at 0300 h. On both day 1 and 2 nitrogen-fixing activity rose for seedlings and fell for trees between 0700 and 1000 h. This is the only major difference in the diurnal patterns between the seedlings and trees. On day 2, which was rainy, no midday drop in activity occurred. Activity rose at midday and continued to rise through the afternoon.

The midday drop in activity on day 1 and increase in activity on day 2 probably reflect photosynthetic patterns for trees on sunny vs cloudy days. On hot, sunny days, excessive water loss from evapotranspiration can cause stomatal closure at midday when light intensity is highest. In contrast, on cloudy days moisture stress is less and a

midday peak in photosynthesis often occurs (Kramer and Kozlowski 1979). Since nitrogen-fixing activity depends on the supply of current photosynthate (Wheeler 1971; Pate 1977; Greig et al. 1962), a dip in activity on day 1 and rise in activity on day 2 are consistent with established photosynthetic patterns for trees.

CONCLUSIONS

Annual, seasonal, and diurnal nitrogen-fixing activity of *I. jinicuil* were quite variable. Year to year variation was large and difficult to explain. Accurate characterization of annual fixation by this or similar species may therefore necessitate very long-term studies.

Monthly fluctuations in nodular activity are considerable and appear caused by the integrated effects of climate and tree phenology. Because peak activity occurred at different times in the two annual reproductive cycles, it may be advisable to sample nodular activity throughout as much of the phenological year as possible. This may be particularly important for tropical evergreens that flower several time annually.

Diurnal activity patterns were pronounced on some days and absent on others. Combining all data yielded a diurnal pattern with a peak at 1900 h and relatively constant rates throughout the rest of the day. Clearly, sampling only at midday or during the daylight hours, as diurnal patterns for most other species would suggest, would miss the peak rate and thereby underestimate daily activity.

Pronounced temporal variation in nitrogen-fixing activity is not confined to *I. jinicuil* but has been documented for most nitrogen-fixing species. However, we found that the timing of maximum and minimum activity, both seasonally and daily, differed in several respects from what has been reported for most species. This suggests that future studies on annual fixation by *I. jinicuil*, or other species with

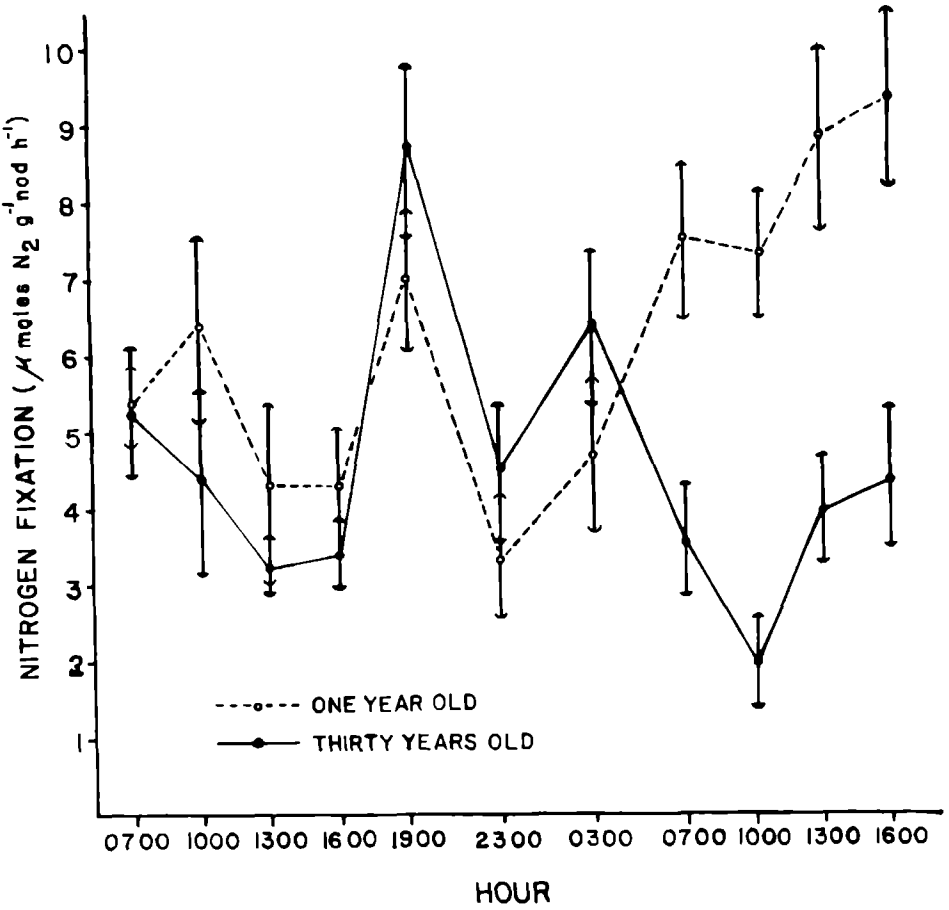


Figure 3. Nitrogen fixing activity for Inga nodules from one-year-old seedlings and 30-year-old trees vs. times of the day. Time sequence goes from 0700 h, September 22 through 1600 h, September 23, 1980. Values shown are means ± 1 S.E.

large temporal variability in nodular activity may have to be of longer duration than is presently customary.

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CHAPTER 5.

Effect of H_2 evolution on $^{15}N_2$-fixation, C_2H_2 reduction and relative efficiency of leguminous symbionts.

ABSTRACT

The $(C_2H_4 + H_{2(C_2H_2)})/^{15}N_2$ ratios of 15 clover-Rhizobium symbionts, soybean, and black medic symbionts were measured. Relative efficiency based on the C_2H_4 production and on ^{15}N-incorporation were compared, and in most symbionts there was little difference between the two measures of relative efficiency. Total measurable electron flux through nitrogenase during acetylene reduction and ^{15}N-incorporation were nearly equal for most symbionts studied. The relative efficiency and the $(C_2H_4 + H_{2(C_2H_2)})/^{15}N_2$ ratio showed an inverse correlation. Use of this ratio appears preferable to use of the ratio of C_2H_2 reduction/N_2 reduction. Some evolution of H_2 was observed in the presence of C_2H_2.

INTRODUCTION

Since the discovery that acetylene is an alternative substance for nitrogenase (Dilworth 1966, Schöllhorn and Burris 1967) it has been used extensively for measuring nitrogenase activity in both laboratory and field experiments (Koch and Evans 1966, Stewart et al. 1967). However, it is necessary to convert the ethylene produced from acetylene to equivalent N_2 reduced to give the measurements meaning in terms of the input of nitrogen into ecosystems by N_2 fixation. A ratio of 3:1 often has been used for acetylene reduced versus N_2 fixed, the ratio being derived from the fact that 6 electrons are necessary for the reduction of N_2 and 2 electrons for reduction of acetylene to ethylene. This ratio assumes no losses in energy from evolution of H_2 or that all H_2 produced by nitrogenase will be oxidized by hydrogenase with all energy obtained being recycled to nitrogenase. As Burris (1974) pointed out, in most experiments the theoretical ratio of C_2H_4/N_2 of 3 is not observed and an appropriate ratio should be established by

measuring C_2H_2 reduction and $^{15}N_2$ reduction under specific experimental conditions.

The theoretical ratio of C_2H_4/N_2 of 3 is not obtained because the inherent production of H_2 by nitrogenase is influenced little by N_2 at the pN_2 usually present during the process of N_2 reduction (Rivera-Ortiz and Burris 1975). ATP is required for the reduction of protons to H_2 (Hadfield and Bulen, 1969, Ljones and Burris 1972). Evolution of H_2 occurs when any hydrogenase present cannot recycle all the H_2 produced. This decreases the efficiency of the nitrogenase and gives a higher C_2H_4/N_2 ratio. The ratio also is increased because acetylene suppresses the production of H_2 much more markedly than does N_2 (Rivera-Ortiz and Burris, 1975). A nitrogenase system with a high relative efficiency (Schubert and Evans 1976) is expected to show a lower C_2H_4/N_2 value than a system with low relative efficiency (Peters et al. 1977). Because measurements of C_2H_2 reduction are less expensive and more convenient that ^{15}N assays, most reported values of relative efficiency have been based on C_2H_2 reduction (Schubert and Evans 1976, Lim 1978, Bethlenfalvay and Phillips 1979, Nelson and Child 1981).

It is assumed that the total electron flux through nitrogenase is constant, independent of the substrate used (Hadfield and Bulen, 1969, Ljones 1973). However, the total electron flux through nitrogenase may decrease with increasing pN_2. With C_2H_2 as substrate, total electron flux decreased somewhat at low pC_2H_2, but at pC_2H_2 = 10 kPa and higher, electron flux was maximal (Hageman and Burris 1980).

We compared the relative efficiency of different clover, soybean, and black medic cultivars based on acetylene reduction assays and on ^{15}N-incorporation. We also determined H_2 evolution/$^{15}N_2$ reduction ratios, $C_2H_4/^{15}N_2$ ratios, and compared the measured electron flux through nitrogenase during acetylene reduction and ^{15}N- incorpora-

tion. There was a correlation between the relative efficiency and the $(C_2H_4 + H_2(C_2H_2))/{}^{15}N_2$ ratio.

Abbreviations--RE, relative efficiency; DW, dry weight.

MATERIALS AND METHODS

Plants and growth conditions.

Soybeans (Glycine max (L.) Merr), red clover (Trifolium pratense L. cv. Arlington, Trifolium pratense L. cv. Kenstar, Trifolium pratense L., from the USSR), white clover (Trifolium repens L.), alsike clover (Trifolium hybridum L.) and black medick (Medicago lupulina L.) were grown in a greenhouse (22°C; winter and spring months; sunlight was supplemented with light from metal arc lamps) in pots filled with autoclaved vermiculite and sand in a 1:1 ratio. The three red clovers, the white clover and the alsike clover were inoculated with a Rhizobium strain (162BB1, 162P17, or 162X6) to give 15 different clover-Rhizobium combinations. Soybeans were inoculated with a native strain isolated from a black medick nodule. A Hoagland's N-free nutrient solution (Hoagland and Arnon, 1938; Fe, 16.67 mg/l as sequestrene 330 Fe, 0.3 mM Fe) was applied twice a week; water was supplied when necessary. Pots were placed on separate trays to avoid cross contamination of the Rhizobium strains.

Analytical methods

At harvest, nodules were separated into two equal parts for the acetylene reduction assays and for ^{15}N-incorporation tests. To avoid transport of fixed ^{15}N, only excised nodules were used. Acetylene was generated by adding CaC_2 to water. Acetylene reduction was initiated by adding 10% by volume of acetylene to the incubation vials. After 40 min subsamples of the gases were taken and analyzed for C_2H_4 and H_2. C_2H_4 was measured by gas chromatography at 75°C with an Aerograph Model 600-D unit,

equipped with a flame ionization detector; N_2 was the carrier gas and Porapak R was the column packing. H_2 was measured gas chromatographically with a Gow-Mac Series 150 unit equipped with a molecular sieve 5A column and a thermal conductivity detector. Argon was the carrier gas and the column temperature was 75°C. ^{15}N-incorporation assays were conducted simultaneously and under the same incubation conditions. After closing the incubation vials with vaccine stoppers, the vials were evacuated for about 10 s through a manifold with a rotary vane vacuum pump. Then a gas mixture of 20% O_2, 0.04% CO_2, 70% argon, and 10% $^{15}N_2$ was added. $^{15}N_2$ gas was obtained from ^{15}N-enriched $(NH_4)_2SO_4$ by adding alkaline NaOBr. ^{15}N-enriched N_2 was mixed successively with alkaline $KMnO_4$ and with 3 M H_2SO_4 to remove residual nitrogen oxides and ammonia, respectively (Burris 1974). After 40 min incubation the gases were sampled. The atom % ^{15}N of the gas mixture was determined with a MAT 250 isotope-ratio mass spectrometer. Because possible leakage of air may have changed the molecular species of N_2 to a non-equilibrium balance of masses 28 ($^{14}N^{14}N$), 29 ($^{14}N^{15}N$), and 30 ($^{15}N^{15}N$), all three molecular species were measured (Burris 1974). Nodules were dried for 48 h at 70°C, weighed and transferred to micro-Kjeldahl flasks. After digestion with H_2SO_4 plus $HgCl_2$, the solution was diluted and powdered Zn was added to amalgamate the Hg. The solution was made alkaline and was steam distilled for 7.5 min into 10 ml of 0.018 M H_2SO_4. Ammonium concentrations were determined with Nessler's reagent (Burris and Wilson 1957). Nodules used for the acetylene reduction assays were digested and distilled the same way and served as controls for the determination of the natural abundance of ^{15}N. Ammonium was converted to N_2 with NaOBr. The R value of the samples (R=Mass29/Mass28) was printed out by a Hewlett Packard 9815-A minicomputer on the mass spectrometer. Atom % ^{15}N was calculated by the formula, atom % ^{15}N= 100R/(2+R). Atom % ^{15}N enrichment

due to fixation was considered as the difference in atom % ^{15}N of the nodules exposed to $^{15}N_2$ and the atom % ^{15}N of the nodules used for the acetylene reduction assays.

RESULTS

Rates of C_2H_4 production, H_2 evolution during the C_2H_2 reduction assays and during ^{15}N-incorporation, and rates of $^{15}N_2$ fixation of the different plant-Rhizobium symbiosis are given in Tab. 1. Although it generally is assumed that no H_2 evolution occurs during acetylene reduction assays (Schubert and Evans, 1976), Bethlenfalvay and Phillips, 1975, Cruch and Tough, 1981), we found H_2 was evolved during the assays. Similar findings have been reported by Rivera-Ortiz and Burris (1975) and by Hadfield and Bulen (1969) with purified nitrogenases of Azotobacter vinelandii, by Peters et al. (1977) with Azolla-Anabaena azollae, by Smith et al. (1976) with Azotobacter, and by Gibson and Alston (1981) with Lupinus angustifolius. At infinite pC_2H_2, no H_2 evolution should occur (Rivera-Ortiz and Burris 1975). C_2H_2 generated from CaC_2 contained H_2 that was easily detected gas-chromatographically, and mass spectrometric measurements confirmed the presence of H_2 in C_2H_2. Values for H_2 evolution by nitrogenase under C_2H_2 equaled 5 to 10% of the values obtained for C_2H_4 production, and about 10% of the values obtained for H_2 production during the reduction of $^{15}N_2$.

Various ratios, e.g., ratios of C_2H_4 to $^{15}N_2$ fixed, and relative efficiency values (Tab. 2), can be calculated from the data in Tab. 1. Because H_2 is evolved during C_2H_2 reduction assays, this H_2 production was added to the C_2H_4 production for calculating the ratios $C_2H_4/^{15}N_2$. More precisely, the $(C_2H_4 + H_2(C_2H_2))/^{15}N_2$ formula

Tab. 1. Rates of reduction of C_2H_2 and $^{15}N_2$ and production of H_2 by various legumes and associated rhizobia. Rates are expressed as μmoles (g DW) $^{-1}$ h^{-1} ± S.E.

Plant species	Rhizobium strain	Replicates n	C_2H_4	$H_{2(C_2H_2)}$	$H_{2(^{15}N_2)}$	$^{15}N_2$
Red clover var. Arlington	162BB1	5	138.0 ± 23.7	9.4 ± 1.8	59.9 ± 14.3	55.9 ± 27.0
Red clover var. Arlington	162P17	4	166.7 ± 47.9	12.0 ± 5.4	73.4 ± 22.1	37.2 ± 12.6
Red clover var. Arlington	162X6	5	141.5 ± 33.5	7.9 ± 4.0	63.9 ± 14.3	18.1 ± 2.6
Red clover var. Kenstar	162BB1	5	133.5 ± 39.9	8.0 ± 3.2	79.3 ± 23.2	21.4 ± 7.6
Red clover var. Kenstar	162P17	4	76.4 ± 12.2	3.8 ± 0.8	31.2 ± 9.8	21.2 ± 7.6
Red clover var. Kenstar	162X6	5	70.8 ± 14.9	2.9 ± 1.0	39.4 ± 12.8	14.2 + 6.5
Red clover var. USSR	162BB1	4	97.4 ± 26.1	8.1 ± 4.5	53.8 ± 17.5	14.4 ± 6.5
Red clover var. USSR	162P17	6	64.4 ± 15.2	3.5 ± 1.3	39.3 ± 12.4	11.2 + 4.0

(Tab. 1 continued)

Plant species	Rhizobium strain	Replicates	C_2H_4	$H_2(C_2H_2)$	$H_2(^{15}N_2)$	$^{15}N_2$
Red clover var. USSR	162X6	4	127.4 ± 51.2	7.7 ± 2.7	81.2 ± 35.1	16.6 + 6.9
Alsike clover	162BB1	4	59.2 ± 19.1	3.5 ± 1.6	34.4 ± 10.1	11.6 ± 4.5
Alsike clover	162P17	5	39.9 ± 6.2	1.6 ± 0.5	21.2 ± 4.9	6.5 ± 1.9
Alsike clover	162X6	5	71.1 ± 11.0	3.2 ± 1.0	40.6 ± 11.0	16.1 ± 4.8
White clover	162BB1	5	71.2 ± 9.8	5.4 ± 1.2	46.1 ± 9.1	13.7 ± 2.4
White clover	162P17	4	116.4 ± 54.6	11.8 ± 7.4	66.0 ± 32.1	21.8 ± 5.6
White clover	162X6	5	110.7 ± 16.6	8.7 ± 2.8	64.8 ± 14.8	19.6 ± 9.1
Soybean	61A84	11	5.7 ± 0.7	0.32 ± 0.07	3.7 ± 0.5	1.1 ± 0.2
Soybean	61A76	12	4.6 ± 0.5	0.15 ± 0.08	2.8 ± 0.4	1.0 ± 0.2
Soybean	61A76	8	5.1 ± 0.8	0.41 ± 0.04	2.1 ± 0.4	1.0 ± 0.1
Black medick	native	3	208.7 ± 39.8	20.8 ± 4.0	94.4 ± 13.0	57.8 ± 1.0

Tab. 2. $(C_2H_4 + H_{2(C_2H_2)})/^{15}N_2$ ratio, electrons balance, $H_2/^{15}N_2$ ratio and the relative efficiency of the various legumes and associated rhizobia.

Plant species	Rhizobium strain	$\frac{C_2H_4+H_{2(C_2H_2)}}{^{15}N_2}$	$\frac{C_2H_4+H_{2(C_2H_2)}}{3\,^{15}N_2+H_{2(^{15}N_2)}}$	$\frac{H_2}{^{15}N_2}$	$RE_{(C_2H_2)}$	$RE_{(^{15}N_2)}$
Red clover var. Arlington	162BB1	2.6	0.65	1.07	0.59	0.74
Red clover var. Arlington	162P17	4.8	0.97	1.97	0.56	0.60
Red clover var. Arlington	162X6	8.2	1.26	3.53	0.57	0.46
Red clover var. Kenstar	162BB1	6.6	0.99	3.70	0.44	0.45
Red clover var. Kenstar	162P17	3.7	0.84	1.47	0.61	0.67
Red clover var. Kenstar	162X6	5.2	0.90	2.77	0.47	0.52
Red clover var. USSR	162BB1	7.3	1.09	3.70	0.42	0.46
Red clover var. USSR	162P17	6.1	0.93	3.51	0.42	0.46

(Tab. 2 continued).

Plant species	Rhizobium strain	$\frac{C_2H_4+H_2(C_2H_2)}{^{15}N_2}$	$\frac{C_2H_4+H_2(C_2H_2)}{3^{15}N_2+H_2(^{15}N_2)}$	$\frac{H_2}{^{15}N_2}$	$RE_{(C_2H_2)}$	$RE_{(^{15}N_2)}$
Red clover var. USSR	162X6	8.1	1.03	4.89	0.40	0.38
Alsike clover	162BB1	5.4	0.91	2.97	0.45	0.50
Alsike clover	162P17	6.4	1.02	3.30	0.49	0.48
Alsike clover	162X6	4.6	0.84	2.50	0.45	0.54
White clover	162BB1	5.6	0.88	3.36	0.40	0.47
White clover	162P17	5.9	0.98	3.03	0.49	0.50
White clover	162X6	6.0	0.97	3.31	0.46	0.48
Soybean	61A84	5.7	0.87	3.40	0.38	0.47
Soybean	61A76	4.9	0.83	2.90	0.39	0.51
Soybean	61A76	5.8	1.10	2.23	0.59	0.57
Black medick	native	4.0	0.86	1.63	0.55	0.65

is employed for the conversion of C_2H_2 reduced to N_2 fixed. This may yield a somewhat higher C_2H_4/N_2 ratio than usually is reported in the literature. Another reason for the higher $C_2H_4/^{15}N_2$ ratios is that the $p^{15}N_2$ of 10 kPa used does not saturate nitrogenase, whereas the pN_2 of about 78 kPa in air approaches saturation (Rivera-Ortiz and Burris 1975). A pN_2 of 10 pKa is 1 to 5 times the Michaelis constant for most N_2-fixing organisms, and they will fix 50-90% as well under a pN_2 of 10kPa as under a pN_2 of 78 kPa (Wilson 1940, Burris and Wilson 1957).

Production of H_2 by nitrogenase seems inherent in the process of N_2-fixation, and no nitrogenase has been found that does not produce H_2 (Burns and Hardy 1975, RiveraOrtiz and Burris 1975). In most leguminous symbionts, only 40 to 60% of the available electrons are used for the reduction of N_2, and the rest are used for the reduction of protons to H_2 (Schubert and Evans 1976, Nelson and Child 1981). Thus, the theoretical ratio of H_2/N_2 of 1, that requires partitioning of 75% of the electrons to N_2 and 25% to H_2 (Burns and Hardy 1975), will not be reached (reduction of 2 protons to H_2 requires 2 electrons, and reduction of N_2 to 2 NH_3 requires 6 electrons). In this study, all the symbionts exhibited ratios of $H_2/^{15}N_2$ greater than 1 (Tab 2.). The lowest value was 1.07 and the highest 4.89. The high evolution of H_2 is particularly clear in the relative efficiency values. The relative efficiency (RE) is defined by Schubert and Evans (1976) as:

$$RE = 1 - \frac{\text{Rate of } H_2 \text{ production in air}}{\text{Rate of } C_2H_4 \text{ production}}$$

The equation is modified somewhat by taking into account the H_2 evolution during C_2H_2 reduction. This provides the following equation:

$$RE = 1 - \frac{\text{Rate of } H_2 \text{ production in air}}{\text{Rate of } C_2H_4 \text{ production} + \text{Rate of } H_{2(C_2H_2)} \text{ production}}$$

With $^{15}N_2$, the RE of the symbiont can be measured with the physiological substrate, i.e., N_2 for nitrogenase. The RE then becomes:

$$RE = 1 - \frac{\text{Rate of } H_2 \text{ production in air}}{3x \text{ Rate of } ^{15}N_2 \text{ fixed} + \text{rate of } H_2 \text{ production in air}}$$

In most species tested, the RE obtained with the C_2H_2 method does not vary greatly from the RE value obtained with the $^{15}N_2$ method (Tab. 2). The largest differences were observed in symbionts in which the measurable electron flux through the nitrogenase during C_2H_2 reduction did not equal the measurable electron flux through the nitrogenase during $^{15}N_2$ reduction.

Most measurements on nitrogenase in vitro show that between 10 and 15% of the electron flux through nitrogenase during acetylene reduction is used for the formation of H_2 (Hadfield and Bulen 1969, Hageman and Burris 1980). Tab. 1 shows similar values for our measurements in vivo. In Rhizobium trifolii uptake hydrogenase was absent or showed only low activity. This leads to the conclusion that the relative efficiency values for the system should be 0.75 or lower. If the experimental RE values, based on $^{15}N_2$-fixation and the related $(C_2H_4 + H_{2(C_2H_2)})/^{15}N_2$ values of the different symbionts tested are plotted, the curve (not shown) suggests an inverse correlation between the RE ard the $(C_2H_4 + H_{2(C_2H_2)})/^{15}N_2$ ratio. The experimental points follow reasonably well the theoretical correlation curve between the RE and the $(C_2H_4 + H_{2(C_2H_2)})/^{15}N_2$ of symbionts possessing no

or a weak uptake hydrogenase.

Assuming that the measurable electron flux through nitrogenase during acetylene reduction or during ^{15}N-incorporation represent the real electron flux through nitrogenase, N_2 fixation can be calculated from the values for C_2H_4 formation and H_2 evolution during C_2H_2 assays and in air. The calculated N_2 fixed should equal:

$$N_2 \text{ fixed} = \frac{C_2H_4 + H_2(C_2H_2) - H_2 \text{ (air)}}{3}$$

DISCUSSION

It is clear from earlier studies (Schubert and Evans 1976, Bethlenfalvay and Phillips 1979, Miller and Sirois 1982) and from this study that the theoretical minimal value of $C_2H_4/N_2 = 3$ is not found experimentally in most leguminous symbionts, because the reduction of protons to H_2 by nitrogenase often utilizes over 50% of the available reductant. The $(C_2H_4 + H_2(C_2H_2))/^{15}N_2$ ratios observed in this study approximate those of Bergersen (1970) for excised soybean nodules (6.6:1). The value (5.6 to 6.0:1) obtained for white clover by Masterson and Murphy (1976) also is very near to our average value of 5.63 from Tab. 2.

The RE value reported here based on C_2H_2 reduction in general agree with results based on studies with $^{15}N_2$. The RE reported by Schubert and Evans (1976) for red clover, white clover, and soybean were similar to ours, but our RE for alsike clover was lower (0.45 to 0.49), than their value of 0.68.

There is a relationship between the RE and the $(C_2H_4 + H_2(C_2H_2))/^{15}N_2$ ratios, but skepticism has been expressed

that symbiotic nitrogen-fixing organism with a high RE have a higher symbiotic effectiveness (plant dry matter or total nitrogen) than those with a low RE. Schubert et al. (1978) found a positive correlation between the RE and the symbiotic effectiveness of soybean (Hup^+ and Hup^- strains tested), but Gibson et al. (1981) reported that soybeans inoculated with Hup^+ or Hup^- R. japonicum strains showed no correlation between the RE and symbiotic effectiveness. When symbionts with an RE near 1.0 show a $(C_2H_4 + H_2\ (C_2H_2))/{}^{15}N_2$ of about 3, this indicated that hydrogenase action supports the nitrogenase activity and presumably increases the efficiency of the nitrogenase system (Emerich et al. 1979, Nelson and Salminen 1982, Pedrosa et al. 1982).

It generally is assumed that during in vivo measurements the total electron flux through nitrogenase is constant and independent of the substrate used. In a system with an active hydrogenase, the total measurable electron flux during acetylene reduction may be larger than the total measurable electron flux during ${}^{15}N_2$ reduction, because the greater production of H_2 by nitrogenase during the ${}^{15}N_2$ incorporation will support greater recycling of H_2 by the hydrogenase than during reduction of acetylene when less H_2 is produced for recycling. There are reports (Smith et al. 1976) that hydrogenase is inhibted by acetylene (not to be confused with C_2H_2 inhibition of H_2 production by nitrogenase). But Emerich et al. (1979), Peterson and Burris (1978), and Houchins and Burris (1981) found little if any inhibition of hydrogenase by acetylene. When no hydrogenase or a weak hydrogenase is present, the total measurable electron flux through the nitrogenase as measured by C_2H_4 + H_2 produced should be equal, within the experimental error, to the measurable electron flux measured by ${}^{15}N_2$ reduction + H_2 evolution. For the symbionts tested, in 17 out of 19 cases, the difference in electron flux measured by

the acetylene reduction assay and by $^{15}N_2$ reduction was less than 20%; flux values for the C_2H_4 production were both higher and lower than electron flux values associated with $^{15}N_2$ reduction.

We saw little difference in electron flux with N_2 vs C_2H_2 as substrate; the differences observed by Hageman and Burris (1980) were in an imbalanced system of purified enzyme components from A. vinelandii. No large differences were reported with lupin (Gibson and Alston 1981) and with isolated Anabaena azollae (Peters et al. 1977). Saito et al. (1980) found a $C_2H_4/(H_2 + 3N_2)$ ratio of 1.32 to 1.43 with nodulated roots of Phaseolus vulgaris. The RE was 0.66 and the H_2/N_2 ratio approximately 2.7, so it seems unlikely that the presence of an active hydrogenase can be invoked to explain these results. For symbionts without uptake hydrogenase, it appears possible to calculate N_2-fixation more exactly by taking into consideration H_2 evolution than by using the arbitrary C_2H_4/H_2 ratio of 3. With adequate replication, the calculated value may equal the value of N_2 fixation measured by ^{15}N-incorporation. An active uptake hydrogenase makes measurements more difficult because hydrogen produced by nitrogenase may be recycled by the hydrogenase during acetylene reduction or during N_2 fixation; the percentage of electrons used for the reduction of protons to H_2 will not be measurable directly.

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CHAPTER 6

Use of ^{15}N-depleted nitrogenous compound for estimation of biological nitrogen fixation.

ABSTRACT

Albizia lebbeck and Leucaena leucocephala, two leguminous trees, were grown in various concentrations of ^{15}N-depleted $(NH_4)_2SO_4$ nutrient solutions. Mass spectrometric analyses did show significant differences in atom % ^{15}N of all leaves and roots tested, grown under different conditions. Experimental use of ^{15}N-depleted $(NH_4)_2SO_4$ for calculation of the percentage of nitrogen derived from seed + N_2-fixation and/or nutrient solution is showed.

INTRODUCTION

Since the heavy, stable isotope of nitrogen, ^{15}N, has been available (Thade and Urey, 1939), there have been numerous reports of the use of this isotope for the estimation of biological nitrogen fixation (Hauck and Bremmer, 1970). In field studies, biological nitrogen fixation has been estimated by application of enriched ^{15}N-salts to the soil and measurement of ^{15}N-incorporation into N_2-fixing plants and non-N_2-fixing control plants; from mass spectrometric and total nitrogen analysis the input of nitrogen from N_2-fixation can be calculated. Because of the high cost of ^{15}N-enriched salts, most field studies have been carried out on small plots with salts of low ^{15}N-enrichment (Ruschel et al., 1982; Wagner and Zapata, 1982).

Because of the higher sensivity of available isotope ratio mass spectrometers (determination of ^{15}N enrichment to 0.005 atom %)it is possible to use ^{15}N-depleted nitrogeneous compounds instead of ^{15}N-enriched compounds. ^{15}N-depleted N compounds are available with around 0.0005 atom % ^{15}N. The natural abundance of ^{15}N in air and soil is around 0.368 atom %, so plants dependent on soil nitrogen or N_2-fixation will show an ^{15}N concentration near 0.368 atom %, so plants dependent on soil nitrogen or N_2-fixation

will show an ^{15}N concentration near 0.368 atom %. Small decreases of this ^{15}N concentration can easily be detected by an isotope ratio mass spectrometer. The main advantage of using depleted ^{15}N-salts is that their lower cost permits the use of relative large experimental plots than those used with ^{15}N-enriched compounds.

This communication reports the use of ^{15}N-depleted $(NH_4)_2SO_4$, applied at different N-levels, to permit calculation of the percentage of nitrogen derived from the seed + N_2-fixation and/or $(NH_4)_2SO_4$ by Albizia lebbeck and Leucaena leucocephala seedlings.

MATERIALS AND METHODS

Seeds of Albizia lebbeck (L.) Benth. and Leucaena leucocephala (Lam.) de Wit (= glauca (L.) (Allen and Allen, 1981), obtained from Dr. Roskoski, INIREB, Xalapa, Mexico, were grown in potts filled with vermiculite and sand in a 1:1 ratio. Albizia plants were inoculated with native Rhizobium strain, earlier isolated from Albizia nodules. L. leucocephala was inoculated with Rhizobium strain 94A3, a generous gift of Dr. Burton, Nitragin Co., Milwaukee, WI, USA. A N-free Hoagland nutrient solution was added twice a week; it contained 16.67 mg Fe/l as Sequestrene 330. In addition twice a week 0, 2.5, 12.5, and 25.0 ml. of ^{15}N-depleted $(NH_4)_2SO_4$ solution (14.7 mMol N concentration, 0.0051 atom % ^{15}N) was added. Additional water was supplied when necessary. At harvest, plants were seperated into nodules, roots, and stems + leaves. The plant samples were digested in micro-Kjeldahl flasks with H_2SO_4 and with $HgCl_2$ as a catalyst. Zn was added to the diluted digests to amalgamate Hg. The digests were made alkaline with 13N NaOH and were steam distilled for 7.5 minutes into 10 ml. of 0.036 N H_2SO_4. Ammonium was determined with Nessler's reagent (Burris and Wilson, 1957). NaOBr was used for converting ammonium to N_2. The R value of the sample (R = M29/M28) was

provided by a Hewlett Packard 9815A computer, connected to the mass spectrometer. The atom % ^{15}N was calculated using the formula: atom % $^{15}N = 100R/(2+R)$.

RESULT AND DISCUSSION

Plants were checked periodically for the presence of nodules. Except for the plants receiving 25 ml. $(NH_4)_2SO_4$ twice a week, all the plants produced nodules. At the time of harvest, however, most of the nodules of A. lebbeck and all the nodules of L. leucocephala were detached and did not show any acetylene reduction activity. A. lebbeck had started forming new nodules at this time whereas L. leucocephala showed no new nodules. Old nodules could not be recovered adequately to give reliable biomass data for nodules.

Table 1. Characteristics of A. lebbeck and L. leucocephala seeds.

Seed	weight in g.	% N	Total mMol N	atom % ^{15}N excess
A. Lebbeck	0.159 + 0.008*	5.06 + 0.15	0.576 + 0.037	+ 0.0023 + 0.0003
L. Leucocephala	0.058 + 0.003	4.60 + 0.13	0.190 + 0.009	- 0.0022 + 0.0002

* Values represent means ± SEM.

Table 1 reports the biomass, % total nitrogen, total mMol N and atom ^{15}N excess of the seeds of A. lebbeck and L. leucocephala. Seeds of A. lebbeck showed an increase in atom % ^{15}N as compared with the atom % ^{15}N of air,

whereas the seeds of L. leucocpehala showed a decrease in atom % excess as compared with air.

Five months after planting the seeds, plants were harvested. Table 2 shows the % total N and the biomass of the stems + leaves and the roots of the two species grown with different concentrations of ^{15}N-depleted $(NH_4)_2SO_4$. Fig. 1 shows the atom percent of ^{15}N of the different plant parts. All values are significantly different at the F = 0.01 level when comparison is made for a plant part of one species grown under different levels of $(NH_4)_2SO_4$; the exception is for the roots of A. lebbeck plants growing with 0 and 2.5 ml. of $(NH_4)_2SO_4$ which are significantly different at the F = 0.05 level. The differences observed between atom % ^{15}N of roots and leaves/stems of the same species can be explained as follows:

a. After 3 months of growth the oldest leaves (which had asimilated N from the seeds) turned yellow and fell off. During this leaf fall, new leaves were being formed, but nitrogen from the seeds was no longer available and the nitrogen necessary for the formation of the leaves had to come from $(NH_4)_2SO_4$ or from N_2-fixation. Plant parts wholly or partly dependent on nitrogen from the nutrient solution would show a decrease in atom ^{15}N. This response is apparent in the newly formed leaves + stems as compared to earlier formed roots. b. During the germination of the seeds and the early formation of the roots and stems/leaves, more nitrogen from the seed is used for the formation of the below ground parts of the plant than for the above ground parts. c. It also may be that the ^{15}N derived from the seed is diluted more in stem/leaves than in the roots because they grew faster than the roots. Because of leaf fall and loses of nodules, it was not possible to measure the total biomass produced by the plants, and the allocation of total N from the seed to the different plant parts could not be calculated accurately.

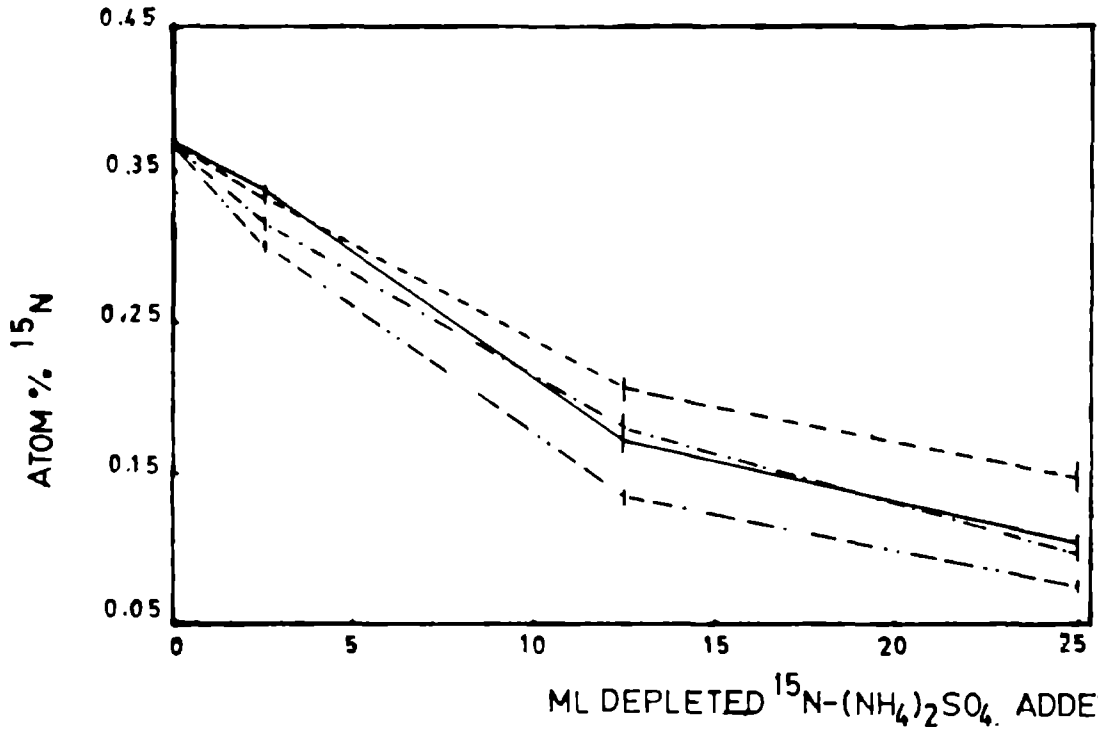

Fig. 1: Atom % ^{15}N of Albizia lebbeck and Leucaena leucocephala roots and leaves, grown under different additions of depleted ^{15}N-$(NH_4)_2SO_4$.
- - - - - - = A. lebbeck roots ———— = A. lebbeck leaves
-.-.-.-.-.- = L. leucocephala roots ..--..--..--..-- = L. leucocephala leaves

Table 2. Biomass and % total N of leaves and roots of A. lebbeck and L. leucocephala grown under different levels of $(NH_4)_2SO_4$.

Albizia lebbeck

ml. $(NH_4)_2SO_4$	Roots		Leaves	
	Biomass*	%N	Biomass	%N
0	0.360 ± 0.039	1.45 ± 0.09	0.320 ± 0.049	2.15 ± 0.03
2.5	0.394 ± 0.580	1.45 ± 0.10	0.307 ± 0.029	2.26 ± 0.13
12.5	0.363 ± 0.027	1.78 ± 0.07	0.332 ± 0.043	3.30 ± 0.16
25.0	0.463 ± 0.038	2.22 ± 0.09	0.357 ± 0.091	4.00 ± 0.21

Leucaena leucocephala

ml. $(NH_4)_2SO_4$	Roots		Leaves	
	Biomass*	%N	Biomass	%N
0	0.204 ± 0.036	1.34 ± 0.03	0.115 ± 0.012	2.60 ± 0.18
2.5	0.170 ± 0.054	1.46 ± 0.03	0.092 ± 0.012	2.58 ± 0.25
12.5	0.217 + 0.024	1.65 ± 0.04	0.136 ± 0.019	3.56 ± 0.11
25.0	0.245 ± 0.036	2.10 ± 0.05	0.170 ± 0.008	3.57 ± 0.12

* Biomass in g dry weight. Values represent the mean ± SEM.

Table 3: Percentages of total nitrogen derived from $(NH_4)_2SO_4$ and seed + N_2 fixation. Percentage of N derived from ^{15}N-depleted $(NH_4)_2SO_4$ are obtained by mass spectrometric analyses, and other values are derived as described in the text. Data are given as % of total nitrogen derived from a specific source.

Albizia lebbeck

		Leaves		Roots	
Rep.	ml. $(NH_4)_2SO_4$ supplied	Seed+N_2 fix.	$(NH_4)_2SO_4$	Seed+N_2 fix.	$(NH_4)_2SO_4$
6	0	100	0	100	0
6	2,5	91.1	8.9	90.4	9.6
6	12.5	46.4	53.6	57.8	42.2
6	25.0	28.4	71.6	39.9	60.1

Leucaena leucocephala

		Leaves		Roots	
Rep.	ml. $(NH_4)_2SO_4$ supplied	Seed+N_2 fix.	$(NH_4)_2SO_4$	Seed+N_2 fix.	$(NH_4)_2SO_4$
5	0	100	0	100	0
5	2.5	83.6	16.4	87.7	12.3
5	12.5	35.1	64.9	47.3	52.7
5	25.0	20.4	79.6	24.3	75.7

Plants receiving 25 ml. $(NH_4)_2SO_4$ twice a week formed no nodules throughout the experiment. Thus, those plants were totally dependent on the nitrogen from the seed and from the nutrient solution. Fig. 1 shows that the atom % ^{15}N of these plant parts was higher than the atom % ^{15}N of the nutrient solution (atom % ^{15}N = 0.0051). The difference was caused by the presence of ^{15}N of normal abundance in the seeds. Because of leaf and nodules fall during plant growth, the total amount of N of the whole plant during the whole growth period is not known. The atom % ^{15}N of the nitrogen derived from seeds or from N_2-fixation will be more or less equal. The percent N derived from seed or from N_2-fixation can, therefore, not be estimated. From mass spectrometer analyses the % nitrogen derived from the nutrient solution can be measured. The rest of the nitrogen is derived from the seed + N_2-fixation (Table 3). The percent of N derived from seeds is, at this stage of plant development, measurable. However, this become small and neglectable with full developed plants.

The use of ^{15}N-depleted $(NH_4)_2SO_4$ gave significant decreases in atom % ^{15}N of plant analyzed. Differences in atom % ^{15}N obtained in this study will be more pronounced than differences in field tests that involve nitrogen from the soil (atom % ^{15}N around 0.368). Deibert et al. (1979) found that between 6 and 37 % of the total nitrogen of nodulated soybeans was derived from fertilizer applied earlier. These values were dependent of the age of the plant harvasted and the amount of N-fertilizer applied. Ruschel et al. (1982) found percentages of 5.5 to 34.0 of N derived from fertilizer dependent on the plant part analyzed, age of the plant and the amount of N-fertilizer applied. If only 5 % of the total nitrogen were derived from ^{15}N-depleted $(NH_4)_2SO_4$ with an atom ^{15}N of 0.0050, the calculated decrease in atom % ^{15}N of the plant material of 0.018 would be detected easily by an isotope-ratio mass spectrometer.

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CHAPTER 7.

Diffusion of gases through plastic bags containing plants being exposed to acetylene or $^{15}N_2$.

INTRODUCTION

Since its introduction the acetylene reduction technique for measuring N_2-fixation (Koch et al., 1966; Sloger et al., 1967; Stewart et al., 1967) has been applied widely. Glass vessels of fixed volume commonly have been used as incubation containers. Plastic bags, however, offer the advantage that when gas samples are removed the internal pressure does not change but adjusts to the external pressure (Burris, 1974). Plastic bags are inexpensive, can be employed in large sizes, and their volume can be adjusted by the amount of gas added. Volume control is important with a valuable gas as $^{15}N_2$. The rates of gas diffusion through plastic films vary markedly and the choice of a suitable plastic is particularly important when incubations are prolonged. The relative and absolute diffusion rates of a number of gases through plastics have been reported (Burris, 1974; Rogers, 1956), but the conditions employed for measurements did not correspond to those encountered when exposing plants to gases. When diffusion was measured, plastic films were placed in a permeability cell and a vacuum was created on one side of the cell. Because of the permeability of the plastic, the difference in pressure between the two sides decreased with time. When the area and the thickness of the film, the temperature and the diffusion constant were known, the permeability could be calculated. But gases such as C_2H_2, H_2 and CH_4 and C_3H_8 were not tested.

I report rates of diffusion of these gases through plastic films under conditions simulating those for measurements of C_2H_2 reductions. I also report the diffusion of $^{15}N_2$ through Saran and polyethylene under conditions similar to those for following $^{15}N_2$ assimilation by N_2-fixing organisms.

MATERIALS AND METHODS

Saran (polyvinylidine) bags and polyethylene bags were used. The collapsed bags were 22 cm. long and 13.5 cm. wide (8.5 cm. diameter inflated) and the thickness was 25 um. Natural rubber stoppers (45 mm. top diameter and 25 mm. long) were used as closures. H_2, C_2H_4, CH_4, and C_3H_8 employed were cylinder gases. C_2H_2 was made by adding CaC_2 pellets to water. Bags were sealed around the stopper with waterproof adhesive tape. An iron wire, 1.5 mm. diam., was tightened over the tape as a second seal to decrease gas leakage around the stopper. To check for leaks in the bags, through and around the stoppers, air was removed with a vacuum pump. If no acceptable vacuum was obtained, the bag or stopper or both were discarded. Once empty, air and other gases were injected with a syringe and needle through the small serum stopper closing the tube that passed through the stopper (Fig. 1). Gas mixture under test consisted of 1 $cm.^3$ CH_4, 2 $cm.^3$ C_2H_4, 10 $cm.^3$ C_2H_2, 4 cm. C_3H_8, 15 $cm.^3$ H_2 and 240 $cm.^3$ air.

The sensivity of the gas chromatographic unit for each gas was determined by measuring the peak height of standards. A Carle g.c. unit (model 9500) with flame ionization detector was used for CH_4, C_2H_4, C_2H_2, and C_3H_8. A Gow Mac g.c. unit with thermal conductivity detector was used for H_2. The composition of the gas mixture in the bag was determined chromatographically, and then the bag was placed in the 12 l round-bottom flask (fig. 1). When the diffusion through the plastic only was to be determined, the bag was placed in the flask as in Fig. 1A. When the diffusion through the plastic and the stopper was to be determined, the bag was placed as shown in Fig. 1B. After a measured time, subsamples of the gas were taken from around the bags through the serum stopper

a, (Fig. 1A and 1B), and their composition was analyzed chromatographically. The difference in gas concentrations between flask A and B represented gas diffusion through or around the stopper. Gas diffusion was calculated by comparing the composition of gases sampled from around the bag and the gas present in the bag. Flasks were flushed with compressed air for 5 min. before starting experiments. After flushing, the atmospheres in the flasks were checked chromatographically for any gas residues. Experiments were replicated with different bags and stopperd. The temperature was about 25°C throughout the experiments.

Diffusion of $^{15}N_2$ was determined by converting NH_4^+ from ^{15}N depleted $(NH_4)_2SO_4$ to N_2 with NaOBr: 60 ml. of ^{15}N-depleted N_2 (about 0.05 atom % ^{15}N) was placed in each bag. Every 30 min. for 4 h for the polyethylene bags and during 8 h. for the Saran bags, the atom % ^{15}N is gas samples from the bags was determined, using an isotope ratio mass spectrometer. The correlation coefficient and the slope of the linear regression line for absolute increase in atom % ^{15}N with time was calculated. The natural abundance of ^{15}N in the air was 0.362 atom %.

RESULTS AND DISCUSSION.

Diffusion through the Saran bag and stopper was relatively slow whereas losses of gases through polyethylene were substantial (table 1 and 2). It should be pointed out that the % diffusion h^{-1} or % increase $^{15}N_2$ h^{-1} is dependent on the surface area of the bag and the partial pressure of the gas within it.

The ratio of diffusion rate of $^{15}N_2$ through Saran and polyethylene (assuming that the diffusion of $^{15}N_2$ is equal to that of $^{14}N_2$) is much smaller (1 : 11.7) than

Table 1. Diffusion of gases through Saran and polyethylene bags closed with a rubber stopper.

Gas	Saran bag	Saran bag and stopper	Stopper	Poly-ethylene bag	Ratio Saran: poly-ethylene
		% loss h^{-1} ± SEM			
Acetylene	0.56 ± 0.06	0.72 ± 0.04	0.16	14.01 ± 0.61	1 : 25.0
Ethylene	0.22 ± 0.02	0.38 ± 0.02	0.16	12.81 ± 0.51	1 : 58,2
Methane	0.16 ± 0.02	0.29 ± 0.02	0.13	6.76 ± 0.24	1 : 42.3
Propane	0.12 ± 0.01	0.26 ± 0.01	0.13	15.59 ± 0.61	1 : 119.9
Hydrogen	1.57 ± 0.05	2.21 ± 0.01	0.64	20.14 ± 0.88	1 : 12.8

Table 2: Diffusion of $^{15}N_2$ through Saran and polyethylene. Values are means ± SEM.

	$r^{1.}$	$a^{2.}$	% increase in $^{15}N\ h^{-1}$
Polyethylene	0.999 ± 0.000	0.0345 ± 0.0043	9.42
Saran	0.959 ± 0.023	0.0029 ± 0.0004	0.80

1. Linear regression coefficients for change in atom % ^{15}N in the bags with time.

2. Slope of regression lines (change in atom % $^{15}N\ h^{-1}$).

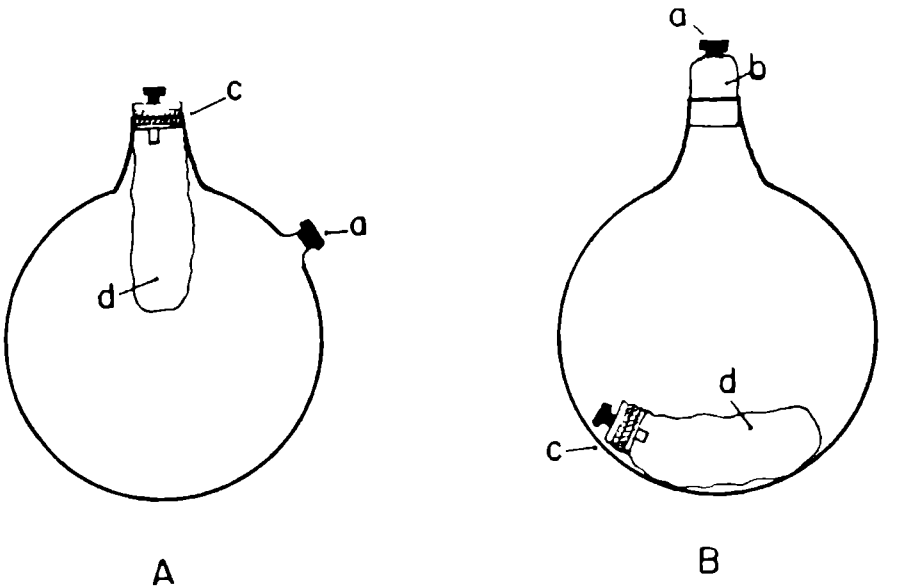

Figure 1.

Round-bottom flasks (12 l) and arrangement of stoppers and bags for estimation of diffusion of gases through plastic films. For further details, see text.

a. Serum stoppers through which subsamples of gases were taken. b. Standard (55 mm. inside diameter, 50 mm. long) glass stopper. c. Rubber stopper, adhesive tape and iron wire to close the bag against the stopper. d. Plastic bag.

the ratio observed by Rogers et al. (1956, 1 : 2020). However, my technique is very different as I sought to simulate conditions of plant exposures in bags. Furthermore, my observations include the effect of sealing the bag around the rubber stopper, and any losses of gases around the stopper and septum stopper. My measurements have been designed for realistic evaluation of diffusion and other losses of gases during the C_2H_2 reduction and $^{15}N_2$ incorporation experiments that have been reported. The results indicate that substantial losses of gases may occur through polyethylene bags of 25 mm. thickness, during measurements of N_2-fixation. Saran is a greatly superior material for the construction of containers for these experiments.

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CHAPTER 8.

^{15}N-natural abundance of N_2-fixing nodules and leguminous plants infected by various strains of rhizobia.

ABSTRACT

The atom % ^{15}N values of nodules of leguminous plants are compared with those of atmospheric N_2. All nodules tested showed higher atom % ^{15}N than N_2 of air. A weak correlation coefficient was found between atom % ^{15}N excess of nodules and activity for $^{15}N_2$-fixation whether or not the nodules transported N as ureides or amides. Nodules from ureide-transporting plants did show a higher atom % ^{15}N excess than did amide transporting nodules: 0.0014 ± 0.0010 vs 0.0010 ± 0.0002 atom % ^{15}N excess, respectively. Significant differences in atom % ^{15}N excess were observed between clover plants inoculated with different strains of Rhizobium trifolii. Furthermore, there were significant differences in atom % ^{15}N excess among the clover species that all were dependent on atmospheric N_2 as their N-source. Problems encountered in using small deviations from ^{15}N-natural abundance for measuring N_2-fixation are discussed.

INTRODUCTION

The variation in natural ^{15}N-abundance in plant tissue and soil was first reported by Hoering (16) in 1955. He showed, for example, that leaves of Trifolium repens had a lower $^{15}N/^{14}N$ ratio than the standard ratio for atmospheric N_2. Delwiche and Steyn (9) suggested that the N-isotopic composition of N_2-fixing plants and other plants could give information about their sources of nitrogen. They found that during N_2-fixation an isotope discrimination occurred, and consequently plants partly or wholly dependent on atmospheric N_2 showed lower atom % ^{15}N than non-N_2-fixing control plants. Because of this discrimination, they suggested it would be possible to determine the % of total N of the plant that had been derived from N_2-fixation. Plants dependent upon soil N should show

a higher ^{15}N concentration than N_2-fixing plants; the effect is enhanced because of soil nitrogen is slightly but variably enriched in ^{15}N as compared with atmospheric N_2 (5, 8, 11, 12, 24).

As non-N_2-fixing control plants, non-inoculated plants (1) or a cereal (23) have been used. A problem with a non-fixing control plant is that its normal ^{15}N abundance may differ from that of leguminous plants that are not fixing N_2. Delwiche et al.(10) showed that different plant families had different $^{15}N/^{14}N$ ratios and that not all N_2-fixing plants produced low $^{15}N/^{14}N$ ratios. This led to the use of isoline plants as controls for the plant species tested, i.e., control plants of the same species as the test plant but with only the difference that the isoline was unable to fix N_2 (18). However, only few non-fixing isolines are available and only for agronomically important species such as Glycine max. An additional problem is that asymbiotic N_2-fixation may benefit the control plant as well the test plant. This N_2-fixation activity may be 10% of symbiotic N_2-fixation (13).

It has been shown that ^{15}N abundance of a N_2-fixing plant is not uniform through the plant and is dependent on the age of the plant (23, 25). The ^{15}N abundance of nodules appears to increase with time when compared with the whole plant (25) whose ^{15}N abundance decreases with time (17). The increase in ^{15}N-abundance of nodules may result from the form of the nitrogenous compound(s) transported from the nodule into the plant, as these may produce an isotopic fractionation within the whole plant. According to Shearer et al. (25) ureide-transporting nodules show a higher ^{15}N-abundance than amide-transporting nodules. Further N_2-fixing efficiency was correlated with ^{15}N-enrichment of soybean nodules (19).

Several investigators (4, 19, 20) have reported the predicted isotope effect during N_2-fixation and/or

transport through the plants, i.e. the heavier ^{15}N atom is discriminated against in favor of the lighter ^{14}N atom. This leads to a lower atom % ^{15}N in an N_2 fixing plant than in atmospheric N_2. Kohl and Shearer (17), however, found an inverse isotope effect with Glycine max and Trifolium pratense, grown hydroponically on an N-free medium. When N_2-fixation measurements are performed with the natural ^{15}N-abundance method, this inverse isotope effect can influence the calculations significantly, especially when observed differences in atom % ^{15}N of test plants, and control plants are small.

It is possible that a specific plant cultivar inoculated with different strains of rhizobia can show different ^{15}N-natural abundance values, even when the same % of total N is derived from N_2-fixation, but no evidence has been reported to support this possibility. When no isolines of the species tested are available or when the use of an isoline is not feasible, non-N_2-fixing control plants of different species must be used. It then is necessary to know whether the ^{15}N-natural abundances of the different control plants are equal. If they differ, it is difficult to make valid measurements of the percentage of the total N derived from N_2-fixation. N_2-fixing plants chosen for comparison should produce the same ^{15}N-natural abundance when the same percentage of their total N has been derived from N_2-fixation.

This paper reports a correlation between the $^{15}N_2$ fixation rate and the ^{15}N concentration of nodules. The effect or different strains of rhizobia on the atom % ^{15}N excess of Trifolium spp. is reported as well as differences in the atom % ^{15}N excess of several Trifolium spp. grown in an N-free medium.

MATERIALS AND METHODS

Soybean (Glycine max (L.) Merr), red clover (Trifolium pratense L. var. Arlington, Trifolium pratense L. var. Kenstar, Trifolium pratense L. from the USSR), white clover (Trifolium repens L.), alsike clover (Trifolium hybridum L.) and black medic (Medicago lupulina L.) were grown in a greenhouse in pots filled with vermiculite and sand in a 1:1 ratio. Sand and vermiculite were autoclaved before use. The three red clover varieties, white clover, and alsike clover were inoculated with one of the following Rhizobium trifolii strains: 162BB1, 162P17, and 162X6. This produced 15 different clover-Rhizobium combinations. Soybeans were inoculated with one of the commercially available Rhizobium japonicum strains 67A84 or 67A76. Black medic was inoculated with a native strain, isolated earlier from a black medic plant. A N-free Hoagland nutrient solution was used, with Fe (16.67 mg./l as Sequestrene 330 Fe, 10% Fe) added, and it was applied twice a week. Additional water was supplied when necessary. Pots were placed on seperate trays to avoid cross contamination of the strains of the rhizobia.

Seeds of Acacia pennatula (Cham. and Schlecht.) Benth., Albizia lebbeck (L.) Benth., Enterolobium cyclocarpum Griseb, Gliricidia sepium (Jacq.) Steud, and Leucaene leucocephala (Lam.) de Wit (= glauca (L.) Benth.) were grown in bags containing approximately 7 kg. of topsoil collected from a pasture at Uxpanapa, Vera Cruz, Mexico. Seeds of Inga jini cuil Schlechter were grown in bags containing approximately 7 kg. of soil, collected from the Botanical Garden of Xalapa, Vera Cruz, Mexico. All seedlings, except I. jinicuil, were placed at the experimental field station of La Mancha, located at sea level near the town of Vera Cruz, Mexico, on the Gulf of Mexico. I. jinicuil seedlings were placed in the Botanical Garden. Water only was supplied as needed. Soybeans were grown for two and a half months, clover and the tropical

leguminous trees, except I. jinicuil, for 4 months. I. jinicuil seedlings were grown for 12 months.

At the time seedlings or plants were harvested, clover plants were separated into nodules, roots and above ground parts (at times, these were divided further into roots and leaves). Nodules were separated from the root system in other plant species. Plant material was dried for 48 h. at 70° C., weighed, and ground (except for nodules) in a Wiley mill through a no. 20 sieve. A subsample of the plant material was transferred to a micro-Kjeldahl flask for digestion with H_2SO_4 and with $HgCl_2$ as a catalyst. Before destillation, powered Zn was added to amalgamete the Hg, and then solution was made alkaline with 13N NaOH; steam destillation was for 7.5 min., and the NH_3 was captured in 10 ml. of 0.036 N H_2SO_4. Ammonium concentrations were determined with Nessler's reagent (7). Controls were included to check for background ammonium. Alkaline NaOBr was used for converting ammonium to N_2. The atom % ^{15}N of the samples was determined with a MAT 250 isotope-ratio mass spectrometer, equipped with a dual inlet and dual collector. The R value of the samples (R = M29/M28) was calculated by a Hewlett Packard 9815-A minicomputer connected to the mass spectrometer, and the atom % ^{15}N was determined from the formula, atom % ^{15}N = 100R/(2+R). Atmospheric N_2 was used as the standard gas.

RESULTS AND DISCUSSION

Table 1 shows the atom % ^{15}N in nodules of N_2-fixing leguminous symbionts as compared with the atom % ^{15}N of atmospheric N_2. None of the values are negative, the lowest recorded value being 0.0000 ± 0.0002 atom % ^{15}N excess for T. repens x Rhizobium 162BB1. The highest value was 0.0065 ± 0.0008 atom % ^{15}N excess for M. lupulina symbiotic system.

Table 1. Rates of $^{15}N_2$-fixation and atom ^{15}N excess of nodules of leguminous symbionts.
Rates are expressed as μmoles hr^{-1} g^{-1} dry wt. ± S.E.
Atom % ^{15}N of atmospheric N_2 is used as standard.

Plant species	Rhizobium strain	Replicates n	$^{15}N_2$	Replicates n	atom % ^{15}N excess
Glycine max	61A76	8	1.0 ± 0.2	8	+ 0.0014 ± 0.0001
Glycine max	61A76	12	1.1 ± 0.2	12	+ 0.0012 ± 0.0001
Glycine max	61A76	3	10.3 ± 3.6	8	+ 0.0025 ± 0.0005
Glycine max	61A84	11	1.0 ± 0.1	10	+ 0.0008 ± 0.0001
Glycine max	61A84	10	2.9 ± 0.3	8	+ 0.0010 ± 0.0001
Medicago lupulina	native	3	57.8 ± 1.0	3	+ 0.0065 ± 0.0008
Acacia pennatula	native	13	3.5 ± 1.0	5	+ 0.0008 ± 0.0003
Albizia lebbeck	native	11	5.9 ± 1.6	5	+ 0.0007 ± 0.0001
Enterolobium cyclocarpum	native	14	4.4 ± 0.9	5	+ 0.0005 ± 0.0001

(Table 1 continued)

Plant species	Rhizobium strain	Replicates n	$^{15}N_2$	Replicates n	atom % ^{15}N excess
Gliricidia sepium	native	11	4.5 ± 1.0	5	+ 0.0020 ± 0.0004
Leucaena leucocephala	native	10	5.3 ± 1.5	5	+ 0.0012 ± 0.0002
Inga jinicuil	native	21	4.6 ± 0.7	5	+ 0.0013 ± 0.0002
Vigna unguiculata	176A22	not measured	not measured	5	+ 0.0029 ± 0.0002
Trifolium pratense var. Arlington	162BB1	6	55.9 ± 27.0	6	+ 0.0012 ± 0.0004
Trifolium pratense var. Arlington	162P17	5	37.2 ± 12.6	5	+ 0.0020 ± 0.0004
Trifolium pratense var. Arlington	162X6	5	18.1 ± 2.6	5	+ 0.0018 ± 0.0005
Trifolium pratense var. Kenstar	162BB1	5	21.4 ± 7.6	5	± 0.0012 ± 0.0006
Trifolium pratense var. Kenstar	162P17	4	21.2 ± 7.6	4	+ 0.0012 ± 0.0006
Trifolium pratense var. Kenstar	162X6	6	14.2 ± 6.5	6	+ 0.0006 ± 0.0003

(Table 1 continued)

Plant species	Rhizobium strain	Replicates n	$^{15}N_2$	Replicates n	atom % ^{15}N excess
Trifolium pratense var. USSR	162BB1	4	14.4 ± 6.5	4	+ 0.0008 ± 0.0004
Trifolium pratense var. USSR	162P17	5	11.2 ± 4.0	5	+ 0.0011 ± 0.0004
Trifolium pratense var. USSR	162X6	4	11.2 ± 4.0	4	+ 0.0021 ± 0.0005
Trifolium hybridum	162BB1	6	11.6 ± 4.5	6	+ 0.0004 ± 0.0003
Trifolium hybridum	162P17	5	6.5 ± 1.9	5	+ 0.0008 ± 0.0003
Trifolium hybridum	162X6	5	16.1 ± 4.8	5	+ 0.0003 ± 0.0002
Trifolium repens	162BB1	5	13.7 ± 2.4	5	0.0000 ± 0.0002
Trifolium repens	162P17	4	21.8 ± 5.6	4	+ 0.0002 ± 0.0001
Trifolium repens	162X6	4	19.6 ± 9.1	4	+ 0.0010 ± 0.0003

Nodules of G. max x Rhizobium 61A76, grown under the same conditions for the same period, but at different times of the year, showed significant differences (P=0.01) in atom % ^{15}N excess. Reasons for the observed differences were not apparant. The various clover symbionts grown on an N-free nutrient medium all showed an enrichment in ^{15}N above atmospheric N_2. The same results were obtained from nodules of the tropical trees A. pennatula, A. lebbeck, E. cyclocarpum, G. sepium, L. Leucocephala, and I. jinicuil. However, the tropical trees were not grown in an N-free medium but in a tropical top soil, so part of the plant's total nitrogen probably was derived from soil N rather than from N_2-fixation. Earlier studies (1, 9, 17, 23) reported that plant parts show an enrichment in ^{15}N when compared with atmospheric N_2, but compared with a non-fixing control plant (e.g., isoline) the N_2-fixing plants show a depletion of ^{15}N. ^{15}N enrichment of soil is reported (11, 12, 23, 24) and this may explain the enrichment in atom % ^{15}N of N_2-fixing plants when compared with atmospheric N_2 but a depletion when compared with non-N_2-fixing plants. Although the observed ^{15}N-enrichment of tropical leguminous nodules may have been caused by the ^{15}N enrichment of the soil, the plants grown on a N-free medium also showed an absolute increase in atom % ^{15}N compared with their available N source, i.e., atmospheric N_2. The mean atom % ^{15}N excess of nodules grown in a N-free medium was 0.0018 ± 0.0004, whereas the nodules grown in soil had a mean value of 0.0011 ± 0.0004 atom % ^{15}N excess. It may be premature to attribute this difference in atom % ^{15}N excess to the different media in which the nodules were produced; the highest ^{15}N values were found in nodules that were completely responsible for providing N for the plant from N_2.

Shearer et al. (26) examined leguminous nodules divided into two classes: nodules transporting N-compounds in the form of ureides and nodules transporting N in the form of

amides to the rest of the plant. Ureides are found in tropical leguminous nodules such as V. unguiculata (3, 15,28) and G. max (3, 21, 27). Trifolium sp. nodules, in contrast, transport N mainly in the form of amides (22). We are not aware of reports concerning the N-compounds which are transported in tropical leguminous trees. Shearer et al. (26) reported that unpublished observations of R. Virginia indicated that the tropical leguminous tree Prosopis glandulosa transports N in the form of ureides. In other tropical legumes, such as Cajanus cajan, Vigna munga, and Vigna radiata, ureides have been found in the xylem sap (3). It often has been assumed that most tropical trees transport N in the form of ureides, but there is little experimental proof for this. We know no evidence on the nature of the N-transport products of M. lupulina. Kohl's (19) hypothesis indicate that nodules of plants transporting N in the form of ureides should be more enriched in ^{15}N than nodules transporting amide N. In this study, the average atom % ^{15}N excess of ureide transporting nodules, i.e. V. unguiculata, A. pennatula, A. lebbeck, G. sepium, E. cyclocarpum, L. leucocephala, I. jinicuil, and G. max was 0.0014 ± 0.0003. The average atom % ^{15}N excess of Trifolium sp. symbiont (amide transporters) nodules was 0.0010 ± 0.0002 atom % ^{15}N excess. Thus, ureide transporting nodules did show a higher atom % ^{15}N excess than amide transporting nodules, but the difference was not as great as reported by Shearer et al. (26). However, Shearer et al. (26) compared the ^{15}N-abundance of nodules with other plant tissues, whereas in this study the comparison was made with atmospheric N_2. We report the differences in atom % ^{15}N between nodules and leaves only for T. pratense var. Arlington, T. hybridum, and T. repens symbionts. In all of these 9 symbiont systems nodules showed an enrichment in atom % ^{15}N. Shearer et al. (26) found a depletion in ^{15}N in T. pratense nodules, but these plants were grown on a nutrient-rich soil in the field or on a nutrient poor soil in a greenhouse.

A correlation between the ^{15}N excess of ureide transporting nodules and their N_2-fixation efficiency (defined as mg N fixed/mg N in the nodules) has been reported (19). The effectiveness of nodules also can be based on their incorporation of ^{15}N. Most of the species reported in this study have been tested for their $^{15}N_2$-fixation (see table 1), and a correlation between ^{15}N-enrichment of nodules and their mode of transport, via ureides or amides, has been established. Correlation coefficients (r) for ureide and amide transporting nodules are 0.65 and 0.52, respectively. This study suggests that a correlation between N_2-fixation activity and atom % ^{15}N excess exists independent of the form in which N is transported from the nodules to other plant parts. Calculation of r for amide transporting nodules by a method similar to that of Shearer et al. (26), gives a value of 0.34; this is possible only for T. pratense var. Arlington, T. hybridum, and T. repens symbionts. No leaves of ureide-transporting plants were analyzed for ^{15}N-abundance. Kohl et al. (19) found a correlation between ^{15}N-enrichment of soybean nodules and N_2-fixing efficiency (r = 0.985). No correlation was found for amide-transporting nodules (26). The results do not contradict each other relative to the correlation coefficient of ureide transporting nodules and atom % ^{15}N. It is important to choose either atmospheric N_2 or some tissue of the nodule-bearing plant as a standard. When atmospheric N_2 is chosen, the absolute difference in atom % ^{15}N vis à vis the N source will be established. However, this standard gives no direct information about ^{15}N-fractionation in the plant. Consequently, ^{15}N-fractionation will give a positive value for atom % ^{15}N excess or a positive r for the atom % ^{15}N excess of nodules; $^{15}N_2$-fixation gives no proof of the occurence of an inverse isotope effect. To estbalish occurence of an ^{15}N-inverse isotope effect requires one to determine the total N of the whole plant.

Table 2 gives the atom % ^{15}N excess over atmospheric N_2 for leaves, roots, and nodules of T. pratense var. Arlington, T. hybridum, and T. repens as affected by strain of the rhizobia. Independent of strain of rhizobia or plant species, all the leaves show a negative atom % ^{15}N excess. Roots showed negative as well as positive values, whereas nodules gave only positive values for atom % ^{15}N excess. The atom % ^{15}N of the nodules gave positive values compared with the leaves. This fractionation of ^{15}N agrees with earlier results (23, 25).

Table 3 shows that all the clover seeds had a ^{15}N "excess" of 0.0001 atom %. Data in tables 2 and 3 give data for calculation of the natural ^{15}N-abundance of the clover plants (Table 4). All the symbionts tested showed a negative value for atom % ^{15}N excess as compared with atmospheric N_2. This indicates a normal isotope effect for clover as has been reported elsewhere for clover and other species (1,9). However, this result disagrees with the data of Kohl et al. (17) who reported that hydroponically grown clover had an atom % ^{15}N greater than the atom % ^{15}N of atmospheric N_2. That is, a slight accumulation of ^{15}N in N_2-fixing clovers occured. When the atom % ^{15}N excess values of all 9 clover symbionts are compared with each other, significant differences are observed (P=0.05) between specific Trifolium species but inoculated with different strains of Rhizobium. Likewise, differences are observed when the different Trifolium species inoculated with a specific bacterial strain are compared; this appears to be independent of the Rhizobium strain used. The discrepancies may lead to errors in measuring the contribution of atmospheric N_2 to the total N in N_2-fixing plants by the natural ^{15}N abundance method. The Rhizobium strain can influence significantly the atom % ^{15}N independent of the amount of N_2 fixed. In this study only atmospheric N_2 was available (except for seed N; it is negligible by harvest time). When a control plant incapable of fixing N_2 was used, differences in atom % ^{15}N between

Table 2. Atom % ^{15}N-excess of nodules, roots, and leaves of various Trifolium - Rhizobium symbionts. As a standard is used atom % ^{15}N of atmospheric N_2. Values represent means ± S.E.

Plant species	Rhizobium strain	Replicates n	Biomass[a]	Total mMol NH_4^+	% N	atom % ^{15}N-excess
Trifolium pratense var. Arlington	162BB1					
leaves		6	2.68 ± 0.56	4.79 ± 1.10	2.45 ± 0.04	- 0.0012 ± 0.0003
roots		6	0.89 ± 0.14	1.12 ± 0.24	1.81 ± 0.09	+ 0.0004 ± 0.0006
nodules		6	43.6 ± 5.8	0.22 ± 0.02	7.18 ± 0.31	+ 0.0012 ± 0.0004
Trifolium pratense var. Arlington	162P17					
leaves		5	2.86 ± 0.58	5.15 ± 1.08	2.88 ± 0.09	- 0.0021 ± 0.0004
roots		5	0.98 ± 0.35	1.36 ± 0.29	2.21 ± 0.17	- 0.0008 ± 0.0001
nodules		5	43.4 ± 5.8	0.22 ± 0.02	7.18 ± 0.31	+ 0.0020 ± 0.0004

(Table 2 continued)

Plant species	Rhizobium strain	Replicates n	Biomass[a]	Total mMol NH_4^+	% N	atom % ^{15}N-excess
Trifolium pratense var. Arlington	162X6					
leaves		5	2.31 ± 0.32	4.03 ± 0.44	2.51 ± 0.15	- 0.0017 ± 0.0003
roots		5	0.68 ± 0.08	0.76 ± 0.12	1.55 ± 0.14	+ 0.0002 ± 0.0003
nodules		5	28.6 ± 2.1	0.13 ± 0.01	6.86 ± 0.33	+ 0.0018 ± 0.0005
Trifolium hybridum	162BB1					
leaves		6	2.65 ± 0.57	4.33 ± 0.87	2.81 ± 0.20	- 0.0014 ± 0.0001
roots		6	1.00 ± 0.29	1.03 ± 0.29	1.85 ± 0.08	- 0.0009 ± 0.0004
nodules		4	38.6 ± 7.9	0.18 ± 0.03	6.48 ± 0.49	+ 0.0004 ± 0.0003
Trifolium hydridum	162P17					
leaves		5	2.10 ± 0.46	3.11 ± 0.35	2.86 ± 0.31	- 0.0010 ± 0.0001
roots		5	0.74 ± 0.22	0.78 ± 0.17	1.91 ± 0.14	+ 0.0019 ± 0.0002
nodules		5	25.5 ± 3.5	0.12 ± 0.01	6.85 ± 0.69	+ 0.0008 ± 0.0003

(Table 2 continued)

Plant species	Rhizobium strain	Replicates n	Biomass[a]	Total mMol NH_4^+	% N	atom % ^{15}N-excess
Trifolium hybridum	162X6					
leaves		5	3.05 ± 0.11	4.44 ± 0.17	3.04 ± 0.17	- 0.0014 ± 0.0001
roots		5	1.16 ± 0.25	1.89 ± 0.38	2.11 ± 0.14	+ 0.0002 ± 0.0009
nodules		5	46.5 ± 10.5	0.21 ± 0.04	6.81 ± 0.46	+ 0.0003 ± 0.0002
Trifolium repens	162BB1					
leaves		6	2.41 ± 0.16	3.84 ± 0.78	3.07 ± 0.15	- 0.0013 ± 0.0002
roots		6	0.75 ± 0.12	0.67 ± 0.09	1.95 ± 0.04	- 0.0003 ± 0.0003
nodules		6	36.5 ± 5.8	0.22 ± 0.02	7.21 ± 0.46	0.0000 ± 0.0002
Trifolium repens	162P17					
leaves		6	3.09 ± 0.36	5.40 ± 1.15	3.13 ± 0.11	- 0.0022 ± 0.0002
roots		6	0.89 ± 0.09	0.92 ± 0.17	1.83 ± 0.12	- 0.0001 ± 0.0002
nodules		6	63.4 ± 8.2	0.31 ± 0.04	7.09 ± 0.27	+ 0.0002 ± 0.0001

(Table 2 continued)

Plant species	Rhizobium strain	Replicates n	Biomass[a]	Total mMol NH_4^+	% N	atom % ^{15}N-excess
Trifolium repens	162X6					
leaves		4	1.72 ± 0.40	3.74 ± 0.97	2.99 ± 0.15	- 0.0020 ± 0.0007
roots		4	0.52 ± 0.14	0.66 ± 0.16	1.71 ± 0.15	+ 0.0020 ± 0.0010
nodules		4	27.8 ± 9.0	0.14 ± 0.05	6.98 ± 0.29	+ 0.0010 ± 0.0003

[a] Leaves and roots values are expressed in g, values for nodules in mg.

Table 3: Atom % ^{15}N-excess of Trifolium seed. As standard is used atom % ^{15}N of atmospheric N_2.
Values represent mean ± S.E.

Species	Replicates n	Biomass in mg	Total mMol NH_4^+	% N	Atom % ^{15}N-excess
Trifolium pratense var. Arlington	4	1.91 ± 0.04	0.00798 ± 0.00024	5.84 ± 0.09	- 0.0001 + 0.0001
Trifolium hybridum	4	0.78 + 0.01	0.00284 + 0.00182	5.31 ± 0.21	- 0.0001 ± 0.0001
Trifolium repens	4	0.75 ± 0.02	0.00292 ± 0.00005	5.22 ± 0.01	- 0.0001 ± 0.0002

Table 4. ^{15}N-natural abundance of nodulated Trifolium species.

Plant species	Rhizobium strain	No. of samples	mMol $^{15}NH_4^+$ excess					atom % ^{15}N-excess[1]
			Leaves	Roots	Nodules	Seed	Total	
Trifolium pratense var. Arlington	162BB1	6	- 64.6 ± 18.6	- 0.8 ± 4.8	+ 2.6 ± 0.9	- 0.002	- 62.9 ± 21.8	- 0.0008 ± 0.0004^{cd}
Trifolium pratense var. Arlington	162P17	5	-103.9 ± 25.6	-11.3 ± 2.7	±41.1 ± 0.0	- 0.002	-111.2 ± 28.2	- 0.0017 ± 0.0003^{ab}
Trifolium pratense var. Arlington	162X6	5	- 70.2 ± 16.6	+ 1.1 ± 2.5	± 2.3 ± 1.0	- 0.002	- 65.4 ± 17.1	- 0.0013 ± 0.0002^{abc}
Trifolium hybridum	162BB1	6	- 64.8 ± 16.7	- 9.9 ± 4.8	+ 0.6 ± 0.4	- 0.003	- 44.2 ± 21.0	- 0.0010 ± 0002^{bcd}
Trifolium hybridum	162P17	5	- 30.0 ± 5.7	+12.9 ± 2.7	+ 0.8 ± 0.5	- 0.003	- 21.7 ± 2.7	- 0.0004 ± 0.0001^{d}
Trifolium hybridum	162X6	5	- 63.5 ± 7.2	- 6.3 ± 13.9	+ 0.6 ± 0.5	- 0.003	- 69.3 ± 20.0	- 0.0010 ± 0.0003^{bcd}
Trifolium repens	162BB1	6	- 58.9 ± 16.6	- 1.7 ± 2.1	+ 0.1 ± 0.0	- 0.002	- 60.6 ± 17.8	- 0.0011 ± 0.0002^{bcd}
Trifolium repens	162P17	6	- 84.7 ± 21.4	- 2.3 ± 2.7	+ 0.6 ± 0.4	- 0.002	- 85.5 ± 23.3	- 0.0012 ± 0.0002^{abc}
Trifolium repens	162X6	4	- 95.0 ± 28.4	- 1.0 ± 5.5	+ 1.6 ± 0.4	- 0.002	- 94.4 ± 43.1	- 0.0018 ± 0.0004^{a}

[1] Values followed by the same letter are not significant different at the 95% confidence level.

the control plant and the N_2-fixing clover plants would indicate different percentages of N were derived from N_2-fixation for different cultivers. However, all clover symbionts derived almost all their N from N_2-fixation. Thus, it is not valid to assume that N_2-fixing plants all show the same ^{15}N-natural abundance when the same % of their total nitrogen has been derived from N_2-fixation.

GENERAL DISCUSSION

The use of deviations in natural ^{15}N abundance of N_2-fixing plants as a measure of N_2-fixation is unexpectedly complicated. Complications include: (a) inverse isotope effects (17) vs. normal isotope effects (9, 20, 23); (b) ^{15}N-fractionation in the plant (17, 23); (c) significant differences in ^{15}N-natural abundance produced by different strains of Rhizobium (this study); (d) significant differences in ^{15}N-natural abundance among clover cultivers fully dependent on N_2-fixation (this study); (e) choice of a proper control plant when no non-fixing isolines of the rhizobia are available (4); (f) interference from variability of the ^{15}N-natural abundance in soils (6, 23, 24). All these factors can affect calculations of the % of total nitrogen derived from N_2-fixation. However, alternative methods such as acetylene reduction, and use of depleted or enriched ^{15}N-salts or ^{15}N-incorporation also have disadvantages. When the ^{15}N-natural abundance method for measurement of N_2-fixation is chosen, a representative subsample of the whole plant should be analyzed for ^{15}N to minimize the effect of ^{15}N-fractionation among the plant parts.

The whole control plant also should be sampled and analyzed for ^{15}N concentration. When no non-N_2-fixing isolines are available, pooled control plants can be used to give a more representative ^{15}N-natural abundance value. Even with these precautions, the ^{15}N-natural abundance method cannot be

expected to provide quantitative information of high accuracy about the source of nitrogen in plants.

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SUMMARY

SUMMARY

Effect of nutrients on nodulation and N_2-fixation by Inga jinicuil, a shade tree in Mexican coffee plantations, was studied. In a field study, high levels of available phosphorus were correlated with high nodule biomass. High levels of soil-nitrogen were correlated with low nodule biomass. In a pot experiment with garden soil as growth medium, effects of fertilizers on nodulation and N_2-fixation by I. jinicuil seedlings was investigated. High levels of phosphorus enhanced nodulation during the first growth period but medium levels of phosphorus increased acetylene reduction rates. Effects of phosphorus fertilization disappeared when the seedlings were 1 year old. Nitrogen fertilization reduced nodulation as well as acetylene reduction rates. Potassium had no effect on nodulation, but low levels of potassium stimulated and high levels decreased acetylene reduction rates. Magnesium and molybdenum did not affect nodulation or nitrogen fixation activity.

Nitrogen fixation activity was measured by means of the acetylene reduction assay. Conversion of the rate of acetylene reduced to the rate of nitrogen fixed had to be established. For six species of tropical leguminous trees, i.e. Acacia pennatula Albizia lebbeck, Enterolobium cyclocarpum, Gliricidia sepium, Leucaena leucocephala, and Inga jinicuil, $C_2H_4/^{15}N_2$ and $H_2/^{15}N_2$ ratios were determined. $C_2H_4/^{15}N_2$ ratios showed values between 2.4 and 4.7. For the $H_2/^{15}N_2$ ratios values between 0.6 and 1.4 were found. No H_2 evolution was detected during the acetylene reduction assay, indicating the presence of Rhizobium Hup^+ strains. The relative efficiency (R.E.) of the nitrogenase system varied between 0.68 and 0.84. Consequently, 16 to 32 % of the reductants present during the reduction of N_2 is used for the production of H_2.

The same ratios as measured for tropical leguminous trees were established for Glycine max, 15 Trifolium

symbionts and Medicago lupulina. $C_2H_4 + H_2/^{15}N_2$ ratios varied between 2.6 and 8.2. $H_2/^{15}N_2$ ratios varied between 1.07 and 4.89. The R.E. varied between 0.74 and 0.38, indicating that for those species between 26 and 62 % of the available reductants during the reduction of N_2 was used for the reduction of protons to H_2. R.E. of the symbionts tested did not vary beyond the experimental error whether based on ^{15}N-incorporation or on acetylene reduction. A correlation was found between $(C_2H_4 + H_2(C_2H_2))/ {}^{15}N_2$ ratio and R.E. This may suggest that a more reliable conversion from C_2H_2 reduced to N_2 reduced can be made without the use of $^{15}N_2$ when H_2 evolution during acetylene reduction and under N_2-fixing conditions is measured.

For the acetylene reduction assay and ^{15}N-incorporation studies, Saran or polyethylene bags as incubation chambers can be used. However, corrections for diffusion of gases should be made. Absolute diffusion of gases through both plastic films under conditions simulating those for measurements of C_2H_2 reduction or $^{15}N_2$-fixation were made. Losses of gases through a Saran bag plus stopper and a polyethylene bag plus stopper for the different gases tested were 0.26 to 2.21 % and 6.76 to 20.14 % per hr, respectively.

The possible use of ^{15}N-depleted $(NH_4)_2SO_4$ for measuring the percentage of total N derived from N_2-fixation was investigated. A. lebbeck and L. leucocephala seedlings, grown in a greenhouse under various $(NH_4)_2SO_4$ concentrations, showed significant differences in atom % ^{15}N for leaves and roots. Percentages of nitrogen derived from seed + N_2-fixation and/or nutrient solution were calculated.

The method for calculating the percentage of total N derived from N_2-fixation and based on the ^{15}N-natural abundance has been studied. Possible effects of different

Rhizobium trifolii strains on ^{15}N-natural abundance of Trifolium spp. was determined. All nodules showed a positive atom % ^{15}N excess as compared with atmospheric N_2. A weak correlation was found between $^{15}N_2$-fixation activity and ^{15}N-natural abundance of the nodules. Roots showed positive as well negative values for atom % ^{15}N excess, whereas leaves only showed positive values. Whole Trifolium plants did show negative values for atom % ^{15}N-excess as compared with atmospheric N_2, indicating a normal isotope effect. However, significant differences in atom % ^{15}N excess between clover plants inoculated with different Rhizobium trifolii strains were observed. Furthermore, clover species, inoculated with the same Rhizobium trifolii strain and grown in an N-free medium, showed significant differences in atom % ^{15}N excess. The practical use of the ^{15}N-natural abundance method for measuring N_2-fixation appears to be a more complicated method than anticipated.

Het effect van voedingsstoffen op wortelknolvorming en N_2-binding bij Inga jinicuil, een schaduwboon in Mexicaanse koffieplantages, werd bestudeerd. In de koffieplantages was een hoog gehalte aan beschikbare phosphorus gecorreleerd met een grote biomassa aan wortelknollen. Een hoog percentage aan bodem stikstof was gecorreleerd met een geringe hoeveelheid aanwezige wortelknollen. In een kas experiment, met tuinaarde als groeimedium, werden de effecten van kunstmeststoffen op wortelknolvorming en N_2-binding bestudeerd. Grote phosphorus giften verhoogden de wortelknolvorming gedurende de beginperiode terwijl middelgrote phosphorus giften de acetyleen reductie activiteiten verhoogden. Het effect van de phosphorus bemesting verdween na een groeiperiode van een jaar. Stikstof bemesting verminderde de wortelknolvorming en de acetyleen reductie activiteit. Kalium had geen effect op de wortelknolvorming maar grote kalium giften verlaagden, en kleine kalium giften verhoogden de acetyleen reductie activiteit. Magnesium en molybdenum hadden geen effect op de wortelknolvorming en acetyleen reductie activiteit.

De stikstof reductie activiteit werd gemeten door middel van de acetyleen reductie methode. De conversie van de hoeveelheid gereduceerde acetyleen naar de hoeveelheid gereduceerde stikstof werd bepaald. Voor 6 tropische vlinderbloemige bomen, Acacia pennatula, Albizia lebbeck, Enterolobium cyclocaropum, Gliricidia sepium, Leucaena leucocephala, en Inga jinicuil, werden $C_2H_4/^{15}N_2$ en $H_2/^{15}N_2$ verhoudingen bepaald. De $C_2H_4/^{15}N_2$ verhoudingen varieerden tussen 2,4 en 4,7. Voor $H_2/^{15}N_2$ verhoudingen werden waarden tussen 0,6 en 1,4 gevonden. Tijdens de acetyleen reductie experimenten werd geen H_2 evolutie waargenomen hetgeen duidde op de aanwezigheid van Hup^+ Rhizobium stammen. De relatieve efficiëntie (R.E.) van het nitrogenase enzym complex schommelde tussen 0,68 en 0,84. Dit houdt in dat tussen de 16 en 32% van de aangevoerde energie tijdens de

reductie van N_2 gebruikt werd voor de reductie van protonen tot H_2.

Dezelfde bepalingen werden uitgevoerd voor Glycine max, 15 Trifolium symbionten en Medicago lupulina. De $(C_2H_4 + H_{2(C_2H_2)})/^{15}N_2$ verhoudingen varieerden tussen 2,6 en 8,2. De $H_2/^{15}N_2$ verhoudingen vertoonden waarden tussen 1,07 en 4,89. De R.E. schommelden tussen de 0,74 en 0,38 waardoor er bij deze species 26 tot 62% van de beschikbare energie tijdens de reductie van N_2 gebruikt werd voor de vorming van H_2. De R.E., hetzij gebaseerd op de acetyleen reductie methode, hetzij op ^{15}N-incorporatie verschilden, met de experimentele marge in acht genomen, niet van elkander. Er werd een correlatie gevonden tussen $(C_2H_4 + H_{2(C2H_2)})/^{15}N_2$ en R.E. Dit zou kunnen wijzen op een meer exacte conversie van gereduceerde acetyleen naar gereduceerde N_2 zonder gebruik behoeven te maken van het isotoop ^{15}N maar met behulp van H_2 evolutie bepalingen tijdens acetyleen reductie experimenten en verder onder N_2 fixerende omstandigheden.

Voor acetyleen reductie en ^{15}N-incorporatie experimenten kan gebruik gemaakt worden van Saran en polyethyleen zakjes als incubatie vat. Daarbij dient dan echter rekening gehouden te worden met eventuele diffusie van de gebruikte gassen door het gebruikte plastic. De absolute diffusie waarden van de gassen werden bepaald onder omstandigheden identiek aan die tijdens het experiment. Het verlies aan diverse gassen bij het gebruik van Saran en polyethyleen zakjes bedroeg respectievelijk 0,26 tot 2,21% en 6,70 tot 20,14% per uur.

Het gebruik van ^{15}N-arme $(NH_4)_2SO_4$ als een mogelijk hulpmiddel voor het berekenen van het percentage totale N verkregen door middel van N_2-fixatie werd onderzocht. A. lebbeck en L. leucocephala werden onder verschillende concentraties $(NH_4)_2SO_4$ in het groeimedium

opgezet. Deze planten vertoonden in bladeren en wortels significante verschillen in atoom % ^{15}N. Het percentage stikstof afkomstig van het zaad + N_2-fixatie en/of groeimedium werd bepaald.

De methode om de natuurlijke concentratie aan atoom % ^{15}N in de plant te gebruiken als een indicatie en bepaling van het percentage van de totale N afkomstig van N_2-fixatie werd bestudeerd. Het mogelijke effect van verschillende Rhizobium trifolii stammen op de natuurlijke concentratie aan atoom % ^{15}N bij Trifolium spp. werd bepaald. Alle wortelknollen vertoonden een positieve waarde voor atoom % ^{15}N exces indien ze werden vergeleken met de atoom % ^{15}N van atmosferische N_2. Een geringe correlatie werd gevonden tussen $^{15}N_2$-fixatie activiteit en de atoom % ^{15}N. De wortels van Trifolium spp. hadden zowel negatieve als positieve waarden voor atoom % ^{15}N terwijl de bladeren alleen negatieve waarden vertoonden. Voor de gehele plant werden alleen negatieve waarden voor atoom % ^{15}N gevonden hetgeen wijst op een normaal isotoop effect. Er werden echter significante verschillen gevonden tussen klaver planten van dezelfde species maar geïnoculeerd met verschillende Rhizobium stammen. Verder werden er significante verschillen gevonden in atoom % ^{15}N tussen klaver soorten geïnoculeerd met verschillende Rhizobium stammen, maar alle afhankelijk van atmosferische N_2 als enige N brom. Het practisch gebruik van deze methode om de N_2-fixatie activiteit te bepalen is gecompliceerder dan werd verondersteld.

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CURRICULUM VITAE

Chris van Kessel, born 1950 at Vorstenbosch, the Netherlands, studied at the College of Agriculture, 's-Hertogenbosch. After receiving his BcS in Agronomy, he worked from 1971 till 1974 as agricultural consultant for the Dutch Government in Cameroun, Africa. In 1974 he started his studies at the University of Nijmegen where he received his BcS in Biology in 1977. In 1978, as part of his graduate work, he investigated, under supervision of Dr. Den Hartog, the ecology of *Zostera* in Sète, France. In the same year he departed for Mexico where, under supervision of Dr. Roskoski and Dr. Linskens, N_2-fixation by *Inga jinicuil* was studied. In 1980, he left Mexico for Madison, U.S.A. where he continued, under supervision of Dr. Burris and Dr. Vogels, his work in N_2-fixation.

I

If a manuscript of W.W.Umbreit, submitted for publication in the late 30's, had been accepted, the importance of ureides in nitrogen transport in nodulated plants would have been recognized earlier.

Umbreit, W.W. 1938 The chemical composition of soybean nodules in relation to the mechanism of nitrogen fixation. Thesis, University of Wisconsin.

Wilson, P.W. 1940 The Biochemistry of Symbiotic Nitrogen Fixation. pg 182-187

II

Apparent opposite results in yield and total nitrogen of leguminous plants inoculated with Hup^+ or Hup^- Rhizobium strains may indicate that, besides recycling H_2, more unknown factors were involved.

Schubert, K.R., Jennings, N., and Evans, H.J. 1978 Plant Physiol. 61:398-401

Gibson, A.H., Dreyfus, B.L., Lawn, R.J., Sprent, J.I., and Turner, G.L. 1981. In A.H.Gibson and W.E.Newton, eds. Current Perspectives in Nitrogen Fixation. Australian Academy of Science. p 373

III

When through genetic engineering non-leguminous plants, such as corn or wheat, would be able to fix N_2, it may not be assumed directly that yield or total biomass would decrease because of the extra energy requirements for biological nitrogen fixation. The theoretical estimates of the costs of nitrate assimilation and of N_2 reduction and assimilation are about equal.

The Energetic of Biological Nitrogen Fixation. 1982 Summary Reports of a Workshop, 1980, at Michigan State University. K.R.Schubert, ed. Published by the American Society of Plant Physiologists.

IV

If diurnal variations in total electron flux through nitrogenase is due to changes in electron flux to protons rather than to N_2, the acetylene reduction assay will become less suitable for measurements of diurnal variations of N_2-fixation.

Rainbird, R.M., Atkins, C.A., and Pate, J.S. 1983 Plant Physiol. 72: 308-312

V

Accuracy in calculating the percentage of total nitrogen derived from biological nitrogen fixation would increase when efforts employing the ^{15}N-incorporation method are positively correlated with efforts employing the acetylene reduction method.

VI

The re-introduction of chinampas (floating gardens) in Mexico as an agricultural production system should be based on soil-hydrological and agricultural studies rather than on historical or nostalgic sentiments.

VII

Hoewel de chinampas geheel onder toezicht stonden en beheerd werden door de Azteken was er eerder sprake van een semi-natuurlijk landschap dan van een gecultiveerd landschap.

Westhoff, V. 1971 In E.Duffey and A.S.Watt eds. The Scientific Management of Animal and Plant Communities for Conservation. pg 3-14

VIII

De doctoraal student dient zich meer bewust te zijn van de mogelijkheden in het buitenland.

IX

The difference between a Republican and a Democratic policy in the White House is only the name.

X

Iedereen wil oud worden, niemand wil oud genoemd worden.

XI

Er dient een einde te komen aan het fiscale-marginale denken betreffende het inkomen en het daaraan gekoppelde inkomstenbelastingstelsel om te voorkomen dat er in Nederland niemand meer werkt.

XII

De grootte van sommige nieuwgebouwde gemeentehuizen doet eerder denken aan toename dan aan afname van de plaatselijke bureaucratie.

XIII

Gezien het vernuft aanwezig in calculators en het summiere gebruik van en begrip voor de potentiële mogelijkheden, dient de prijs van telmachientjes veelvoudig vermenigvuldigd te worden.

XIV

Het beste vooruitzicht op een zonnige dag in de ochtend is nog steeds het veelvuldig aantreffen van verkeerscontroles op de Nederlandse wegen.

XV

Bij de huidige discussie omtrent de kruisraketten dient niet zozeer de aandacht gevestigd te worden op de plaats van stationering dan wel op de plaats waar ze terecht komen.

XVI

Het vermelden van de Nederlandse afkorting 'Drs.' in Engelstalige tijdschriften betekent een pretentieus gebruik van de Engelse titulatuur en dient derhalve vermeden te worden.

XVII

Na de invoering van het democratiseringsproces in Nederland zouden veel vergaderingen staande gehouden dienen te worden.

C.H.J. van Kessel, 1983